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THE JOURNAL OF GENERAL MICROBIOLOGY

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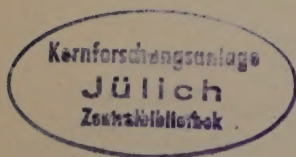
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Modification of the Biological Effects of Ultraviolet Irradiation by Post-irradiation Treatment with Iodoacetate and Peptone

With an appendix on an interpretation of the effects of post-irradiation treatments based on a chemical model

BY S. D. WAINWRIGHT* AND ANN NEVILL

Biology Branch, Atomic Energy of Canada Ltd, Chalk River, Ontario

SUMMARY: A study has been made of the effects of post-irradiation treatment of ultraviolet irradiated spores of *Streptomyces* sp. strain T12 with distilled water, iodoacetate and a peptone medium.

At doses exceeding 400 ergs/mm.² (which exceed that corresponding to the peak of the dose/variant-frequency curve) treatment in distilled water results in increases in both the proportion of survivors and the frequency of variants amongst the survivors. Treatment with iodoacetate caused larger increases, whereas treatment with peptone medium suppressed the increases. A partial resolution of the lethal and mutagenic effects of the radiation was obtained at lower doses, for it was possible to modify the proportion of survivors without changing the variant frequency.

Preliminary studies (Wainwright & Mullaney, 1953) have shown that the extent of survival of ultraviolet irradiated cells of *Escherichia coli* could be increased by a brief period of post-irradiation treatment with iodoacetate, an inhibitor of carbohydrate metabolism. In further studies of these effects we have employed spores of *Streptomyces* sp. strain T12, in which the frequency of radiation-induced variants has been found to be exceptionally high (Newcombe, 1953). A partial resolution of the lethal and mutagenic actions of ultraviolet light has been effected, and it would appear that the post-irradiation metabolism of the spores may be of major importance in determining the extent of the observed effects of a given dose of irradiation.

METHODS

All experiments were performed with suspensions of spores of *Streptomyces* sp. strain T12 freshly prepared from plates inoculated with standard suspensions and incubated at 28° for 5 days. The spores were suspended in distilled water, agitated in a blender for 30 sec. to ensure complete dispersion, and freed of adhering mycelial mat by filtration through sterile Whatman no. 2 filter-paper. For experiments with washed suspensions the spores were washed once with sterile distilled water immediately before filtration.

A G.E. germicidal lamp was used for the irradiation and doses were measured with a Westinghouse dose meter. All operations subsequent to the irradiation were performed by the light of red lamps to prevent photo-reactivation.

* National Research Council (Canada) Fellow 1952-3. Atomic Energy of Canada Ltd Fellow 1953-4.

4 ml. Samples of irradiated suspension were diluted with 1 ml. of distilled water or reagent solution and incubated, usually for 3 hr. at 37°. The suspensions were then diluted, plated on standard medium and incubated at 28°. The maximal number of colonies could be observed after 3 days of incubation at 28°, but plates were incubated for 5–7 days to facilitate the scoring of variants.

Estimates of either the proportion of surviving spores or the proportion of induced variant colonies made from plates containing more than eighty colonies per plate were lower than estimates from less crowded plates. Therefore, wherever possible, estimates both of proportion of survivors and of induced variant frequency were made from eight to ten replicate plates each containing between ten and eighty colonies. Usually this condition could be satisfied and all estimates for a given series of treated suspensions made at the same dilutions. In experiments where estimates could not be made from the same dilutions the same qualitative effects of treatment with a given agent were shown by counts made for two dilutions of each of the suspensions.

The basal peptone medium had the composition: K_2HPO_4 0.5 g., asparagine 0.5 g., 'Bacto' peptone 0.5 g., water to 1 l. The standard medium used for plating consisted of basal peptone medium supplemented with 0.5 % (w/v) glucose and 1.5 % (w/v) agar.

Types of variant colony

The most frequently observed types of variant have been described (Newcombe, 1953) and classified according to variations in the quantity of aerial mycelium and in the colour of the mycelial mat.

In addition, we have repeatedly recovered two types of 'pink' variant which differ from both the wild type and the previously described 'red' variant in their response to the inclusion of arabinose in the standard medium in place of glucose. The latter two types give rise to colonies of an intense scarlet colour in the presence of arabinose. This scarlet coloration was not obtained if arabinose was replaced by gluconate, xylose or any other carbon source tested, nor was it obtained by variations in the pH value of the medium. The pink variants yield pink colonies with either arabinose or glucose as carbon source. Although the biochemical basis for these phenomena is not known, they serve to demonstrate that the pink variants represent a distinct class and differ from the red variants in more than a quantitative difference in the amount of red pigment produced. One type of pink variant yields colonies of an intense pink hue under all conditions of growth tested. The second type gives colonies of a light hue when grown as discrete colonies, but gives a growth indistinguishable from that of the wild type when grown under crowded conditions.

Other hitherto undescribed classes of variant included (a) 'golden' and (b) 'watery'. The former yield colonies with both aerial mycelium and mycelial mat of an intense golden hue. These colonies prove to be unstable, for they give a mixture of golden and 'white' variant colonies when subcultured. Watery colonies show a diminished quantity of aerial mycelium, are usually white or pale yellow in colour and superficially appear to be mucoid. These colonies are, however, of the firm texture typical of the *Streptomyces* mycelium.

RESULTS

When spores irradiated at a dose of 400 ergs/mm.² were incubated with 2×10^{-4} M-iodoacetate (neutralized to pH 7.2) the extent of survival was from 2 to 3 times greater than for control irradiated spores similarly treated in distilled water (Table 1). This effect of iodoacetate treatment has been repeatedly observed (e.g. Tables 2 and 3). The extent of the difference in survival varied from as little as 20 % (in one exceptional experiment) to sevenfold, but usually was in the range of two- to threefold.

Table 1. *Influence of treatment with 2×10^{-4} M-iodoacetate and with peptone + arabinose medium upon survival and frequency of variants*

Expt. no.	Control		Treated		
	Distilled water	Plated with iodoacetate	Iodoacetate	Medium*	Medium* iodoacetate
1					
Survival $\times 10^6$	16	14	34	10	10
Percentage variants	39.5	34.8	44.5	34.4	30.1
Counts	71/180	43/124	274/617	57/166	50/166
2					
Survival $\times 10^6$	152	140	343	78	117
Percentage variants	53.1	48.5	58.1	42.5	47.6
Counts	114/215	97/199	255/437	47/111	79/166
3					
Survival $\times 10^6$	11	12	35	11	13
Percentage variants	53.1	50.7	62.9	37.2	28.6
Counts	99/186	92/182	299/479	61/164	61/213

* Basal peptone medium (see methods) plus 10^{-3} M-arabinose.

Dose: 400 ergs/mm.². Temp. 37°. Time of treatment 3 hr.

The counts are the actual numbers of colonies (variants/total) from which the percentage variants was estimated.

Results obtained with washed spore suspensions were no more marked, nor more quantitatively reproducible than with unwashed spores (Table 2). We have, therefore, assumed that the variations in the initial proportions of survivors with unincubated spores and in the extent of the effect of iodoacetate upon survival were due to uncontrollable variations in the initial physiological condition of the unirradiated spores or in the initial viable count of the unirradiated suspension (and, hence, the dose of radiation received per spore). As these quantitative variations have not masked the qualitative effects studied, no attempt has been made to standardize either of the variables at the expense of the other.

We have also consistently found that the proportion of variant colonies arising from iodoacetate-treated spores was greater than with the control irradiated spores incubated in distilled water (Tables 1 and 2). This difference in variant frequencies was highly significant. In the first eighteen of our experiments involving iodoacetate treatment the difference in variant frequency was significant at the 5 % level in fifteen, the sum of individual χ^2 was 246 (18 D.F., $P < 0.001$) and the χ^2 of the pooled data was 248 (1 D.F., $P < 0.001$).

These effects of iodoacetate upon the proportions of survivors and upon the variant frequencies could not be attributed to the small concentrations of iodoacetate carried with the spores on to the plating medium, unless firmly

Table 2. *Influence of treatment with 2×10^{-5} M-iodoacetate and with peptone medium upon survival and frequency of variants*

Expt. no.	Temperature of treatment		Control		Treated		
			0 hr.*	Distilled water	Iodoacetate	Medium	Medium + iodoacetate
1	37°	Survival $\times 10^6$	—	154	214	126	120
		Percentage variants	—	34.0	44.6	24.6	21.4
		Counts	—	81/238	120/269	61/247	42/196
2	37°	Survival $\times 10^6$	—	52	122	32	35
		Percentage variants	—	36.4	49.5	23.5	29.4
		Counts	—	153/421	492/1021	60/256	83/283
3	37°	Survival $\times 10^6$	—	123	390	45	58
		Percentage variants	—	47.4	58.5	37.4	41.8
		Counts	—	68/144	267/456	197/527	286/679
4†	37°	Survival $\times 10^6$	7.4	12	21	9.5	10
		Percentage variants	23.5	32.5	41.1	24.1	24.7
		Counts	107/456	242/745	529/1292	141/584	160/647
5†	37°	Survival $\times 10^6$	36	46	77	47	40
		Percentage variants	19.6	23.8	37.4	19.6	23.5
		Counts	29/148	45/189	110/295	38/194	39/165
6	28°	Survival $\times 10^6$	15	17	54	13	20
		Percentage variants	33.6	44.0	50.2	31.9	41.1
		Counts	65/194	93/211	312/621	30/94	93/226
7	28°	Survival $\times 10^6$	15	22	56	17	18
		Percentage variants	34.7	32.2	58.9	31.6	28.7
		Counts	40/117	63/195	260/444	46/146	45/157
8†	28°	Survival $\times 10^6$	4	7	52	5	7
		Percentage variants	57.4	59.1	69.5	59.2	57.8
		Counts	102/178	174/295	162/234	128/216	191/330
9†	28°	Survival $\times 10^6$	23	27	55	26	33
		Percentage variants	21.1	28.6	44.1	22.4	24.1
		Counts	42/198	107/373	332/753	79/354	109/453

* Control suspension plated immediately after irradiation and dilution.

Dose: 400 ergs/mm.². Time of treatment 3 hr.

The counts are the actual numbers of colonies (variants/total) from which the percentage variants were estimated.

† Experiments with washed suspensions.

Table 3. *Influence of iodoacetate concentration during treatment upon survival and variant frequency*

Expt. no.		Control (distilled water)	Treated with iodoacetate at	
			2×10^{-4} M	2×10^{-5} M
1	Survival $\times 10^6$	32	95	45
	Percentage variants	44.7	64.8	53.5
	Counts	259/581	113/174	481/902
2	Survival $\times 10^6$	165	244	180
	Percentage variants	18.1	31.7	19.8
	Counts	37/205	96/302	44/222

Dose: 400 ergs/mm.². Temp. 37°. Time of treatment 3 hr.

The counts are the actual numbers of colonies (variants/total) from which the percentage variants was estimated.

bound. Plating control irradiated spores incubated in distilled water on agar containing equivalent concentrations of iodoacetate (10^{-7} to 10^{-8} M) did not result in increased survival or variant frequency (Table 1).

Two hundred and thirty-seven variant colonies were isolated from spores treated with iodoacetate, and all were found to retain their variant character upon subculture. If the increased proportion of variant colonies resulting from iodoacetate treatment had been due to the induction of temporary physiological variations of character some forty of these isolates would have been expected not to show a heritable modification of character ($P < 0.001$). Treatment with iodoacetate had not caused any significant effects upon survival or the proportion of spontaneous variants (which was of the order of 1.2 %) with unirradiated spores ($0.3 < P < 0.5$ for 66,000 colonies).

After incubation with peptone medium the extent of survival and the proportion of induced variants were lower than with irradiated control spores incubated in distilled water (Tables 1 and 2). Further, the presence of peptone medium appeared to inhibit the effects of post-irradiation treatment with iodoacetate (Tables 1 and 2). The effects of the medium were more marked in the absence of added carbohydrate. Treatment with the peptone medium had no significant effects upon survival or the proportion of spontaneous variants with unirradiated spores ($P > 0.9$ for 54,000 colonies).

The actions of iodoacetate and of peptone medium appeared to be the enhancement and the inhibition, respectively, of phenomena taking place with spores incubated in the total absence of added nutrient (Table 2).

The effects of incubation for 3 hr. with arabinose (in the absence of peptone), at a concentration of 10^{-3} M, were inconsistent.

Some effects of post-irradiation treatment with 2×10^{-4} M iodoacetate were found with a period of incubation of only 15 min. Survival was increased, from 1.1 to fourfold, in ten of twelve experiments and in two no change was observed ($P < 0.05$ by the sign test). Effects upon the proportions of variants were inconsistent and differences significant at the 5 % level were observed in only two experiments. In those cases in which there was more than a twofold increase in survival the proportion of variants was reduced, and in the remaining experiments (including those in which there was no effect upon survival) the proportion of variants was increased.

Effect of temperature of incubation

Results obtained after treatment at 28° were essentially similar to those observed after post-irradiation treatment at 37° . With the exception of one experiment, the effects observed after 3 hr. incubation were of the same magnitude at both temperatures (Table 2). Treatment with iodoacetate at 28° for 15 min. did not increase the survival above that observed with control spores similarly treated in distilled water in six of eight experiments, and only caused a 25 % increase in survival in the remaining two. On the other hand, iodoacetate raised the variant frequency above that of the corresponding controls in all but one of the experiments, and the increase was significant at the 5 % level in three ($P < 0.001$ for pooled results).

Effect of iodoacetate concentration

The effects of two concentrations of iodoacetate upon spores irradiated at a dose of 400 ergs/mm.² and incubated at 37° are illustrated in Table 3. Iodoacetate was found to have some activity at a concentration of 2×10^{-5} M, but less than at 2×10^{-4} M. Essentially similar results were also obtained for spores irradiated at a dose of 200 ergs/mm.², and for spores irradiated at either dose and incubated for 15 min. Iodoacetate was lethal to unirradiated control spores at a concentration of 2×10^{-3} M, causing extensive killing in 15 min. at 37°.

Effect of dose of irradiation

A dose of 400 ergs/mm.² exceeds that corresponding to the peak of the 'dose variant-frequency' curve (Wainwright & Nevill, 1955). Hence, an increased survival associated with an increased variant frequency as a result of post-irradiation treatment could be the consequence of a reduction in the 'effective' dose of radiation received by the spores. Therefore, the effects of post-irradiation treatments upon spores irradiated at other doses were studied.

Similar results to those obtained with spores irradiated at 400 ergs/mm.² were also obtained with spores irradiated at 600 ergs/mm.² Results obtained with spores irradiated at 200 and at 100 ergs/mm.² are given in Table 4. The effects upon survival were all qualitatively the same as with spores irradiated at the higher doses, except that treatment of spores irradiated at 100 ergs/mm.² with peptone medium did not cause any reduction below the level obtained with controls incubated in distilled water. The effects of incubation in distilled water or with peptone medium upon the proportion of induced variants with spores irradiated at 200 ergs/mm.² were qualitatively the same as with spores irradiated at the higher doses, but were quantitatively smaller. The variant frequency increased after incubation in distilled water (Table 4, columns 4 and 5, χ^2 for pooled data 11.53, $P < 0.001$) and this increase was inhibited in the presence of peptone medium (Table 4, columns 5 and 7, χ^2 for pooled data 7.39, $P < 0.007$). Treatment in either distilled water or peptone medium did not cause any change in the variant frequency in the case of spores irradiated at 100 ergs/mm.². Iodoacetate treatment had no effect upon the proportion of induced variants with spores irradiated at either 200 or 100 ergs/mm.². The effects of the various treatments upon spores irradiated at different doses are summarized in table 5.

DISCUSSION

It is improbable that the effects of the various post-irradiation treatments studied can be attributed to increases in cell numbers due to growth of the spores occurring during the period of treatment and subsequent fragmentation of the mycelium during plating. We have not detected any significant increases in the viable counts of unirradiated control suspensions. Indeed, cytological examination has shown that development of the germination tube is barely initiated after 3 hr. incubation in nutrient medium at 37°. Further, less than 2% of unirradiated control spores germinated in the absence of added

nutrient during the course of incubation for 96 hr. (Wainwright & Nevill, 1955).

It would seem equally improbable that the effects upon the proportions of variant colonies can be attributed to effects upon the germination and growth

Table 4. *Influence of treatment with 2×10^{-4} M-iodoacetate and with peptone medium upon survival and frequency of variants with spores irradiated at doses of 200 and 100 ergs/mm.²*

Expt. no.	Ultraviolet dose (ergs/mm. ²)		Control		Treated		
			0 hr.*	Distilled water	Iodoacetate	Medium	Medium + iodoacetate
1	200	Survival $\times 10^2$	1.6	3.7	8.2	1.3	1.5
		Percentage variants	37.6	44.6	47.5	36.6	44.8
		Counts	114/303	306/688	72/152	86/238	114/255
2	200	Survival $\times 10^2$	0.8	1.9	3.7	0.7	1.0
		Percentage variants	26.4	45.7	37.2	31.8	44.9
		Counts	27/102	131/286	237/637	44/138	85/190
3	200	Survival $\times 10^2$	3.2	5.9	12.2	2.7	3.1
		Percentage variants	32.2	33.3	39.3	32.5	30.9
		Counts	149/462	287/862	70/179	115/354	138/447
4	200	Survival $\times 10^2$	1.7	4.2	5.6	1.3	1.6
		Percentage variants	37.2	40.0	39.4	40.3	38.9
		Counts	277/745	74/185	58/147	231/573	280/720
5	200	Survival $\times 10^2$	2.4	4.4	9.6	—	—
		Percentage variants	34.4	35.9	40.2	—	—
		Counts	71/207	123/342	327/813	—	—
6	200	Survival $\times 10^2$	2.6	5.7	12.0	—	—
		Percentage variants	34.0	38.5	36.4	—	—
		Counts	164/481	357/932	79/218	—	—
7	200	Survival $\times 10^2$	1.8	3.8	—	2.5	—
		Percentage variants	38.0	43.4	—	33.4	—
		Counts	95/250	237/546	—	117/351	—
8	100	Survival $\times 10^2$	43.5	59.2	65.8	55.3	53.0
		Percentage variants	17.1	16.9	17.2	20.2	20.0
		Counts	159/928	214/1272	242/1405	214/1059	227/1133
9	100	Survival $\times 10^2$	39.6	35.4	59.3	40.8	52.6
		Percentage variants	25.7	25.4	24.1	25.7	24.4
		Counts	109/423	95/375	152/631	116/433	136/558
10	100	Survival $\times 10^2$	14.5	46.9	47.2	47.9	45.8
		Percentage variants	23.4	23.3	22.4	17.1	21.0
		Counts	34/145	110/469	93/415	82/479	85/405
11	100	Survival $\times 10^2$	24.8	38.2	52.0	39.1	27.6
		Percentage variants	21.9	25.1	27.1	26.8	27.6
		Counts	70/318	100/398	165/610	134/498	72/260

* Control suspension plated immediately after irradiation and dilution.

Temp. 37°. Time of treatment 3 hr.

The counts are the actual numbers of colonies (variant/total) from which the percentage variants was estimated.

upon the plates (due to traces of reagent carried over during plating) resulting in selection favouring either variant or non-variant types of mycelial growth. The concentrations of iodoacetate present in the plating medium were inadequate to account for the effects observed (Table 1), unless these resulted

from iodoacetate firmly bound to the spores. Moreover, results obtained with irradiated spores treated with iodoacetate and subsequently washed indicate that this reagent is not firmly bound to the spores (Wainwright & Nevill, 1955). Further, the magnitude of the effects of post-irradiation treatment with iodoacetate are dependent upon the duration of the treatment for periods up to at least 96 hr. (Wainwright & Nevill, 1955), whereas the binding of a firmly adsorbed chemical would be expected to be complete well within 3 hr. The amounts of peptone medium carried over to the plates are negligible in comparison with that already present in the plating medium.

Table 5. *Summary of effects of incubation with iodoacetate and peptone medium*

Dose (ergs/mm. ²)	Water*	Iodoacetate†	Medium†
(a) On survival			
400 }	Increased	Increased	Reduced
600 }			
200	Increased	Increased	Reduced
100	Increased	Increased	Unchanged
(b) On frequency of variants			
400 }	Increased	Increased	Reduced
600 }			
200	Increased	Unchanged	Reduced
100	Unchanged	Unchanged	Unchanged

* Effects relative to control spores plated immediately after irradiation and dilution.

† Effects relative to control spores incubated with water.

The results obtained in this study (Table 5) do not correspond to a simple modification by post-irradiation treatment of the 'effective' dose of radiation received by the spores. With spores irradiated at doses exceeding that corresponding to the peak of the 'dose variant-frequency' curve (400 ergs/mm.²), there is indeed a close parallel between the effects of incubation in distilled water or with iodoacetate and of a decrease in radiation dose. Conversely, the effects of incubation in peptone medium correspond to those produced by an increase in the dose of radiation employed (Wainwright & Nevill, 1955). However, with spores irradiated at lower doses some portion of the lethal effect of the radiation can be reversed without any accompanying change in the frequency of induced heritable variants amongst the survivors. Thus, our studies demonstrate a partial resolution of the lethal and mutagenic effects of ultraviolet radiation.

Various possible mechanisms can be proposed to account for the effects of post-irradiation treatments with chemical agents in modifying the biological responses of irradiated cells. At one end of the range are simple chemical mechanisms in which the added chemical can modify a reaction between a hypothetical radiation-produced poison and a sensitive cell component as a result of combining with either of them. At the other end of the range of mechanisms are those which postulate that the added chemical changes the course of the abnormal cell metabolism resulting from the radiation because

of some mechanism known to affect normal cell metabolism (i.e. the added chemical is a metabolic inhibitor, a cell nutrient, the prosthetic group of a known enzyme). Even these extremes do not represent mutually exclusive alternatives, for it is possible to postulate that the poison of the former type of mechanism is an inhibitor of cell metabolism or a degraded substrate or enzyme. There is no limit to the number of hypothetical radiation-produced poisons which may be postulated. Further, there is no limit to the properties which may be ascribed to any one such poison in order to account for the fact that with two reagents of closely similar chemical structure one is active in modifying the effects of the radiation and the other is not. In addition, the only evidence that any applied chemical has actually entered the treated spores is the observation of a definite effect, for it is not generally possible to determine chemically which of the individual spores contain the chemical and in what concentration.

For these reasons it is not possible to provide critical evidence which would eliminate any of the various hypotheses which may be advanced. Rather, we must inquire whether any observed effect of post-irradiation treatment with a chemical reagent is due primarily to the known biochemical properties of the reagent, or to some relatively simple chemical reaction(s) involving a specific type of radiation-produced poison. This means that we must decide: (a) whether the effects of a particular post-irradiation treatment can be explained on the basis of the known biochemistry of the organism and the reagent used, and (b) how complex must be the postulates required to account for the effects by a predominantly chemical model. We may also note that with our questions posed at this level it is immaterial whether any observed effect upon the induced variant frequency is due to a preferential action of the agent leading to greater survival of potentially-variant than of non-variant spores (or vice versa), for such preferential effects would in themselves be due either to an effect upon cell metabolism or to some relatively simple chemical reaction.

The increased survival resulting from incubation in distilled water can be attributed to the replacement of material inactivated during the irradiation through the metabolism of the endogenous reserves of the spores. Any disturbance of the balance of that metabolism, either by the addition of a nutrient (i.e. peptone medium) or a metabolic inhibitor (i.e. iodoacetate), or by dilution of the suspension and plating on solid medium, would be expected to result in a modification of the observed effects of the irradiation. Further, unless the relative extents of inactivation of all the cell constituents were the same at all doses of irradiation, qualitative variations in the effects of treatment with a given agent would be expected at differing doses. Similarly, the effects of the post-irradiation treatments used in modifying the frequency of induced variant colonies can be explained on the basis of effects of the added chemicals upon normal cell metabolism.

Any model required to explain the results obtained in this study (Table 5) by a simple set of chemical reactions has to attribute very complex properties to the hypothetical radiation-produced poisons. We have indicated in the Appendix the postulates which we believe to be the minimum requirements.

Further studies of: (a) the response of ultraviolet irradiated spores given post-irradiation treatment in distilled water or iodoacetate for varying periods of time (Wainwright & Nevill, 1955), (b) of the responses to post-irradiation treatments with other agents of known significance to the cell economy (in preparation), and (c) of the influences of post-irradiation treatments upon the phenomenon of photoreactivation (in preparation), all indicate much more complex minimum sets of postulates.

We, therefore, prefer to interpret our results as the consequences of disturbances of post-irradiation cell metabolism by the added reagents. Thus, they strongly indicate that greater attention should be given to the possible importance of the post-irradiation cell metabolism than has hitherto been accorded to it. The studies of Lwoff and co-workers (see Lwoff, 1952, 1953; Jacob, Siminovitch & Wollman, 1952) have clearly demonstrated the significance of post-irradiation cell metabolism in the phenomena of radiation-induced lysogenesis and colicinogenesis. In the study of other aspects of the action of irradiations, however, critical evidence is not yet available. Nevertheless, reports have been made of phenomena which can be at least as readily interpreted as due to effects upon the post-irradiation metabolism of irradiated cells as by any other mechanism. For example, it has been found that variations of the temperature of post-irradiation incubation cause significant changes in both the observed extent of survival and the frequency of induction of mutations with irradiated cells of *Escherichia coli* (Stapleton, Billen & Hollaender, 1952; Witkin, 1953). Similarly, it has been found that incubation of irradiated cells of this organism with yeast or meat extracts causes increased survival (Hollaender, Stapleton & Billen, 1953). Further, it may be noted that an increased frequency of mutants has been observed with spores of *Trichophyton mentagrophytes* irradiated at a high dose of ultraviolet and incubated in saline for prolonged periods (Hollaender & Emmons, 1941).

If the post-irradiation cell metabolism is of importance in determining the observed effects of the irradiation it might markedly affect the form of the observed 'dose-survival' and 'dose-mutation' curves. Indeed, it is not possible to obtain such curves without permitting considerable metabolism and cell division to occur. Thus, it would appear that conclusions concerning the mechanism of action of irradiations based upon dose-survival (or dose-mutation) curves obtained after treatment of irradiated cells with agents of significance to the cell economy, or after modifications of the environmental conditions known to affect markedly the course of cell metabolism, should be accepted with reserve.

We are greatly indebted to Dr H. B. Newcombe for his stimulating criticism and encouragement at all stages of this investigation.

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APPENDIX

*Interpretation of the effects of post-irradiation treatments
based on a chemical model*

Incubation in peptone medium prevented the increase in the proportion of survivors which occurred when spores irradiated at doses exceeding 200 ergs/mm.² were incubated in distilled water. However, treatment with peptone medium did not prevent the increase in proportion of survivors which occurred when spores irradiated at a dose of 100 ergs/mm.² were incubated in distilled water. Thus, we may, for example, conclude that the concentration of lethal 'poison' produced at a dose of 100 ergs/mm.² is inadequate for appreciable reaction with the peptone to occur (i.e. the concentration is less than the required 'threshold concentration'). Alternatively, we may conclude that peptone medium modifies a chemical reaction involving a lethal poison which is only produced when the dose of radiation exceeds 100 ergs/mm.². Similarly, we may conclude either that the various reagents can only react with a mutagenic poison when the concentration exceeds a certain threshold, or that one mutagenic poison is not formed at doses less than 100 ergs/mm.² and a second mutagenic poison is not formed at doses less than 200 ergs/mm.².

As incubation in distilled water of spores irradiated at doses of 100 ergs/mm.² results in an increased proportion of survivors without any change in the frequency of induced variant colonies we must postulate a minimum of two poisons. If we also postulate that both poisons are lethal and mutagenic, and that interaction with the added reagents only occurs when the concentration of poison exceeds a different threshold concentration for each added reagent,

it is only necessary to postulate this minimum number of two poisons. On the other hand, if we postulate that interaction with the added reagents occurs at all concentrations of the various poisons we must postulate a minimum of two lethal and three mutagenic 'poisons'. The lethal and mutagenic effects of doses of radiation exceeding 200 ergs/mm.² which can be modified by post-irradiation treatments could be those of a single lethal and mutagenic poison, but the results obtained with spores irradiated at doses of 200 and 100 ergs/mm.² (Table 5) require that we postulate a minimum of two distinct mutagenic poisons but only one lethal poison.

We, therefore, believe the minimum postulates required for the interpretation of the effects of the post-irradiation treatments summarized in Table 5 by a relatively simple chemical mechanism to be either: (i) that the irradiation causes the production of two lethal and/or mutagenic poisons, with differing threshold concentrations required for interaction with endogenous cell constituents, peptone and iodoacetate, respectively: or (ii) that four different poisons are produced at different doses, and that there are no threshold concentrations required for interactions with added chemicals. These poisons would be, respectively, one lethal and mutagenic poison, one other lethal poison, and two other mutagenic poisons not identical with either lethal poison.

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Induction of Provariants as a Function of Dose of Ultraviolet Radiation

With an appendix on the contribution of differential effects upon survival of provariant and nonvariant spores in the modifications of induced variant frequency resulting from post-irradiation treatments

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SUMMARY: With spores of *Streptomyces* sp. strain T12 irradiated at doses of ultraviolet light up to 1000 ergs/mm.² and then incubated in distilled water, both the proportion of survivors and the proportion of induced variants amongst the survivors rose progressively to maximal values as the period of incubation was increased. The maximal proportion of variants was attained before the maximal proportion of survivors. The rate of increase of survival and the final level of survival were enhanced in the presence of iodoacetate. The rate of increase of variant frequency was enhanced by iodoacetate, but the maximal proportion of variants was not raised. The changes of variant frequency resulting from post-irradiation treatments do not appear to be due to selectively greater increases in the survival of variant spores than of non-variant spores.

We have recently demonstrated (Wainwright & Nevill, 1955) that post-irradiation treatment of ultraviolet irradiated spores of *Streptomyces* sp. strain T 12 with iodoacetate or a peptone medium can modify both the extent of survival and the proportion of colonies showing radiation-induced heritable modifications of character. The effects of post-irradiation treatment with iodoacetate of spores irradiated at doses of radiation exceeding 400 ergs/mm.² were similar to the effects of a small decrease in the radiation dose. We have, therefore, attempted to determine whether independent modifications of the lethal and mutagenic effects of the radiation could be obtained with spores irradiated at these higher doses. A detailed study of the influence of the duration of the post-treatment has revealed modifications of the proportions of survivors without accompanying changes in variant frequency.

In addition, the nature of the relationship between the maximal proportion of variants obtained after suitable post-treatment and the dose of radiation received has been determined.

METHODS

Preparation of the spore suspensions of *Streptomyces* sp. strain T 12 and the experimental methods used are given in the accompanying paper (Wainwright & Nevill, 1955).

Cytological studies were made with spore preparations stained by the method of Robinow (1945).

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RESULTS

Stimulation of the conversion of provariants into variants*

Examination of the data obtained in our earlier study of the effects of post-irradiation treatment with iodoacetate upon spores irradiated at a dose of 400 ergs/mm.² (Wainwright & Nevill, 1955) suggested that the higher the proportion of variant colonies obtained from irradiated control spores in-

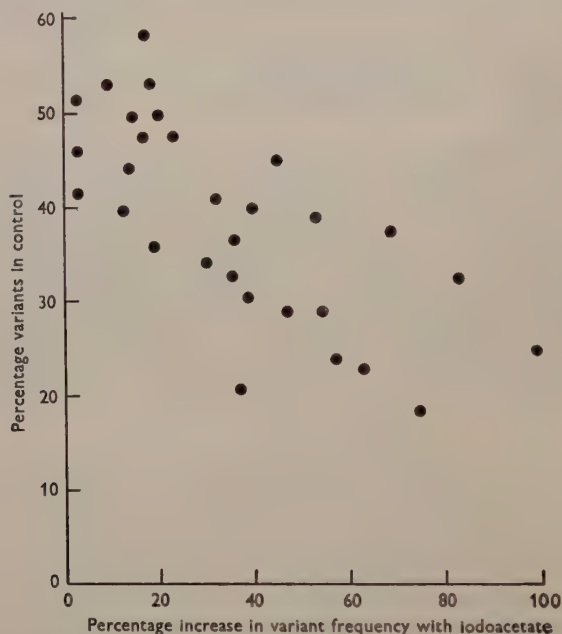


Fig. 1. Enhancement of variant frequency by iodoacetate. Spores irradiated at a dose of 400 ergs/mm.² were diluted 4:5 with either distilled water or 2×10^{-4} M-Na iodoacetate (neutralized to pH 7.2) and incubated for 3 hr. at 37°. The variant frequency in the control suspension (distilled water) is plotted as ordinate. The increase in variant frequency due to treatment with iodoacetate, expressed as a percentage of the control value, is plotted as abscissa.

cubated in distilled water the smaller the effect of treatment with iodoacetate. The results of these and subsequent experiments are illustrated in Fig. 1. Similar inverse correlations have also been found (a) between the increase in proportion of variants induced by treatment with iodoacetate and the initial

* Our results indicate that ultraviolet radiation induces an unstable condition within a proportion of the irradiated spores. Under certain conditions these spores revert to stable non-variant spores and under other conditions these spores become stable variants. We therefore require a definition which will describe such modified spores.

Induced variant colonies can only arise from spores in which the radiation has induced the potential ability to express a heritable variation of character. However, the genotype of any given spore can only be determined after growth and development of a visible colony. The term 'provariant' is, therefore, used to describe those spores which are potentially able to give rise to a variant colony. This definition contains no implication concerning either the genotype or the viability of such spores immediately after cessation of the radiation.

proportion of variants observed with spores plated without post-treatment immediately after irradiation (zero-hour controls), and (b) between the increase in proportion of variants due to incubation in distilled water (3 hr. controls) and the initial proportion of variants (zero-hour controls).

The extent of the increases in variant frequencies resulting from treatment either in distilled water or with iodoacetate were not correlated with the corresponding increases in survival. Some of the most marked increases in survival were associated with small increases in the proportion of variants, and vice versa (see Wainwright & Nevill, 1955). Further, there was no correlation between the extent of the increase in survival due to treatment with iodoacetate and either the initial proportion of survivors from control unincubated spores or the final proportion of survivors from control spores incubated in distilled water. Similarly, the extent of the increase in proportion of survivors following incubation in distilled water could not be correlated with the proportion of survivors from unincubated control spores.

The effect of peptone and other N-sources in suppressing the increase in variant frequency (Wainwright & Nevill, 1955 and unpublished experiments) could not be correlated with the proportions of variants observed with control spores either before or after incubation in distilled water. No correlations were found between the effects of treatment with N-sources upon variant frequency and upon survival.

Influence of length of post-treatment

The effects of post-irradiation incubation for varying periods of time, with iodoacetate or in distilled water, upon the proportions of survivors and of induced variants among spores irradiated at 400 ergs/mm.² are illustrated in Fig. 2.

Three phases have been observed in the recovery of spores incubated in distilled water. The first, or lag, phase was characterized by a progressively rising rate of increase in viable count and was usually completed within 3–5 hr. (Fig. 2*a*). In the second phase the rate of increase in viable count was fairly constant and in the third phase the rate of recovery declined until a maximal level of survival was attained. There was some variation in the lengths of the various phases with different suspensions, and the lag period was frequently not observed (Fig. 2*b*). In the presence of iodoacetate no lag phase in the recovery process was ever observed, and the rate of increase in viable count was markedly raised (Fig. 2*a*). However, at the time of completion of the second phase of recovery in the control suspension the iodoacetate ceased to affect survival, and the subsequent rates of increase in viable count were approximately the same in the presence and absence of iodoacetate (Fig. 2*b*).

The proportion of spores yielding variant colonies observed after incubation in distilled water increased steadily at an approximately constant rate to reach an approximately constant maximal value after 24–48 hr. treatment. This maximal proportion of variants was attained before the maximum level of survival. Similar results were obtained with spores irradiated at doses

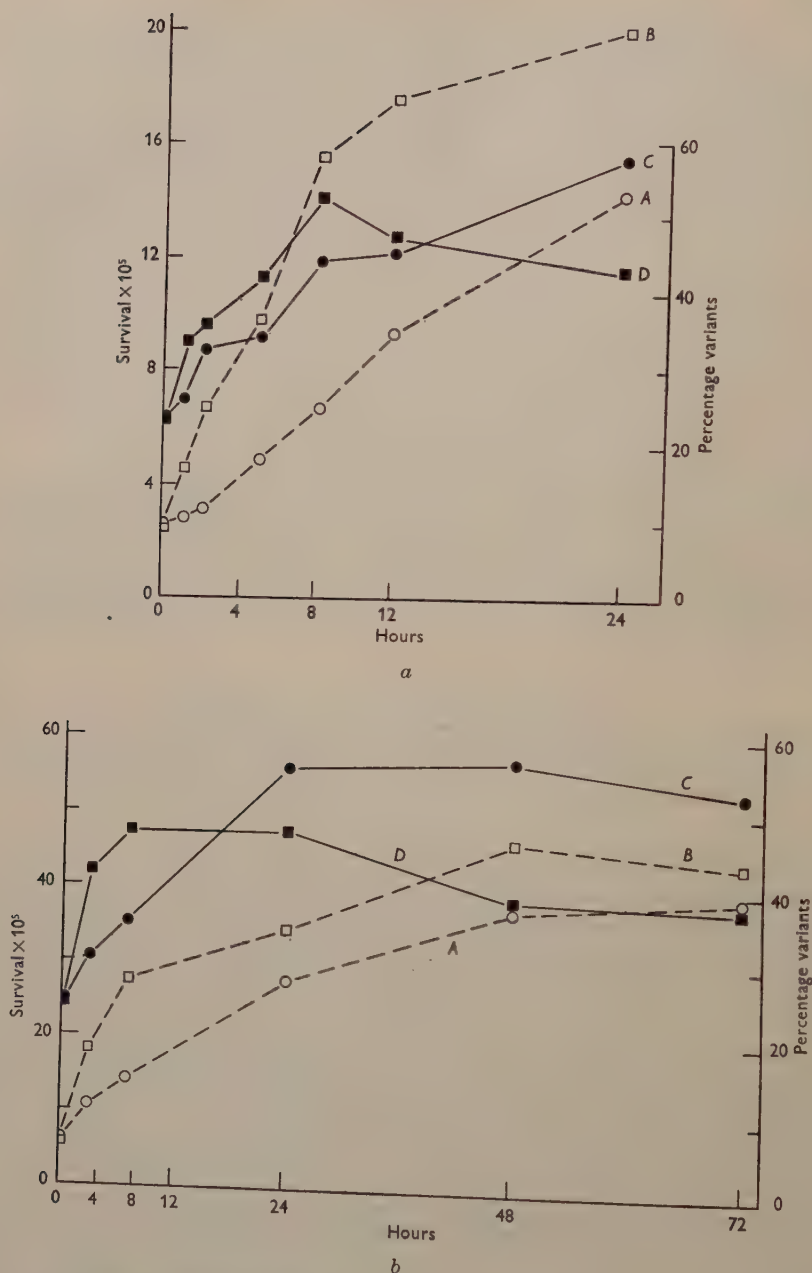


Fig. 2. Influence of post-irradiation treatment with and without iodoacetate. Spores irradiated at 400 ergs/mm.² were diluted 4:5 with either distilled water or 2×10^{-4} M-Na iodoacetate (neutralized to pH 7.2) and incubated at 37°; at intervals samples were plated. Broken lines: survival $\times 10^5$. Solid lines: % variants. Curves A and C: controls, distilled water. Curves B and D: 2×10^{-4} M-iodoacetate. Figs. a and b represent two experiments of this kind.

exceeding 400 ergs/mm.² In the presence of iodoacetate the initial rate of increase in the proportion of variants was enhanced. However, stimulation of the increase in variant frequency stopped at a time coincident with the cessation of the stimulatory effects of iodoacetate upon survival, and thereafter the agent caused a progressive decline in variant frequency (Fig. 2*b*).

Experiments were performed in which portions of the irradiated spore suspensions were washed once with distilled water after a 3 hr. period of post-treatment, then resuspended in distilled water and re-incubated. The maximal proportion of variants obtained with spores treated with iodoacetate and subsequently washed was insignificantly different from that obtained with either washed or unwashed control spores incubated in distilled water (Table 1). As we were unable to prevent some loss of spores, it has not been possible to study in detail the effects of washing upon the course of subsequent increases in survival. However, it was noted that the rate of increase of survival of iodoacetate-treated spores was slightly increased after removal of the agent, whereas the rate of recovery of the spores incubated with distilled water was reduced to approximately one-half as a consequence of washing.

Table 1. *Influence of washing on maximal proportion of variants after post-treatment*

Spore suspensions irradiated at 400 ergs/mm.² were diluted 4:5 with either distilled water or 2×10^{-4} M-Na iodoacetate (neutralized to pH 7.2) and incubated at 37°. After 3 hr. incubation portions of the suspensions were washed once with distilled water, resuspended in distilled water, and reincubated.

Maximal proportion of variants after post-treatment			
Control (distilled water)		2×10^{-4} M-iodoacetate	
Unwashed	Washed	Unwashed	Washed
60.8 ± 2.1	58.9 ± 2.6	56.8 ± 2.7	63.0 ± 4.5
65.6 ± 4.8	60.2 ± 2.5	50.7 ± 1.9	63.1 ± 4.9

No increase was observed in the viable count of unirradiated control suspensions over the period of these experiments. In fact, there was a slight progressive decrease which became significant after 72 hr. incubation. Further, there was no significant change in the proportion of spontaneous variants, which was of the order of 1–2 %. Hence it seems most improbable that either the increases in survival or in the proportions of variants can be attributed to germination of the spores, followed by subsequent fragmentation of the mycelium. Indeed, cytological examination of unirradiated control suspensions incubated for 72 hr. in either distilled water or 2×10^{-4} M-iodoacetate solution revealed that not more than 2 % of the spores had germinated, and for this small proportion of the spores the average number of nuclei present in the resulting mycelia was only 4.

The effects of post-irradiation incubation in distilled water or with iodoacetate upon spores irradiated at 200 ergs/mm.² are illustrated in Fig. 3. In distilled water there was a steady increase in both the viable count and the proportion of induced variants during the course of the 6 hr. period of incubation.

The rate of increase in viable count was raised for a brief period in the presence of iodoacetate. No significant effect of the agent upon the variant frequency was observed during the first 4 hr. of treatment, and thereafter it appeared to cause a reduction in the extent of the increase in the proportion of variants.

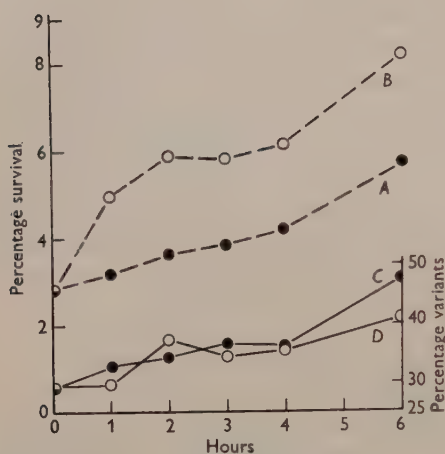


Fig. 3

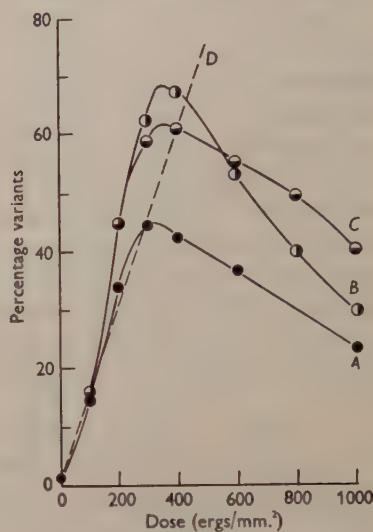


Fig. 4

Fig. 3. Influence of post-irradiation treatment with and without iodoacetate. Spores irradiated at 200 ergs/mm.² were diluted 4:5 with either distilled water or 2×10^{-4} M-Na iodoacetate (neutralized to pH 7.2) and incubated at 37°. At intervals samples were plated. Broken lines: % survival. Solid lines: % variants. Curves A and C: controls, distilled water. Curves B and D: 2×10^{-4} M-iodoacetate.

Fig. 4. Influence of post-irradiation treatment upon the dose/mutation curve. Irradiated spore suspensions were diluted 4:5 with distilled water and incubated at 37°. Curve A: suspensions plated immediately after irradiation and dilution. Curve B: suspensions plated after incubation for 24 hr. Curve C: suspensions plated after incubation for 48 hr. Curve D is the theoretical linear curve to be expected from the proportion of variants obtained at a dose of 100 ergs/mm.².

Influence of post-irradiation treatment upon the dose-survival and dose-mutation curves

It is usually assumed that radiation-induced mutations occur independently of each other, i.e. that the probability of a mutation taking place within any one cell is not affected by a prior mutation within that cell. In addition, it is usually assumed that the extent of the immediate response to irradiation of the cells (e.g. production of toxic chemical intermediates, induction of unstable states in the genes) is a linear function of radiation dose. If the lethal effects of the radiation not due to lethal mutation could be neglected, the shape of a curve showing induced mutant, or variant, frequency as a function of radiation dose could be predicted from these two assumptions. At very low radiation doses it would be expected that the curve would tend to be linear. However, as the dose increased so would the proportion of mutations which

would occur in cells in which a prior mutation had already occurred. Thus, the curve would actually show a less than linear increase in mutant frequency with increase of radiation dose.

The dose mutation (or variant) curves we have obtained experimentally deviate considerably from this theoretical curve. Usually the curve shows a peak (Fig. 4), the mutant frequency rising to a maximum and then decreasing again as the radiation dose is increased. Further, the initial increase in mutant frequency with increase of radiation dose is usually less than would be anticipated from a calculated theoretical curve based upon the frequency of mutants obtained at a low dose of radiation.

It is not our intention to discuss the various hypotheses which may be advanced to account for the shape of dose/mutation curves determined experimentally. Nor do we wish to illustrate the manner in which any one particular set of postulates can be used to account for the shape of curves obtained by experiment. Instead, we wish to note that one postulate which we have made is that the cell components responsive to the mutagenic activity of the radiation (and intermediary products) become 'saturated' at some intermediate dose of radiation.

Taking this postulate in conjunction with the assumptions previously noted, the theoretical dose/mutation curve would be expected to show a plateau. The inverse correlations noted between the extent of the effects of the post-treatments upon the induced variant frequency and the initial proportion of induced variants observed with unincubated controls suggested that the dose/mutation curve obtained after sufficient post-irradiation treatment to permit the attainment of the maximal variant frequency might correspond to this latter theoretical curve.

Table 2. *Influence of dose and post-treatment upon proportion of variants*

Samples of spore suspension irradiated at 400 ergs/mm.² were diluted 4:5 in either distilled water or 2×10^{-4} M-Na iodoacetate (neutralized to pH 7.2) and incubated at 37°. The maximal proportion of variants observed is compared with the initial proportion at 0 hr. treatment, and with the proportion of variants obtained after irradiation of the same stock suspension at 100 ergs/mm.² and plating after treatment in distilled water for 3 hr.

Expt. no.	Maximal proportion of variants				
	Dose 100 ergs/mm. ² (A)	Dose 400 ergs/mm. ² , with post-treatment in			Ratio B:A
		No treatment (0 hr. control)	Distilled water (B)	2×10^{-4} M- iodoacetate	
1	15.2 ± 1.1	33.5 ± 2.7	63.5 ± 3.6	60.4 ± 3.0	4.2
2	13.7 ± 0.9	42.6 ± 5.1	52.2 ± 2.4	47.8 ± 4.4	3.8
3	16.9 ± 3.6	36.8 ± 3.1	65.9 ± 3.8	—	3.9
4	15.3 ± 1.8	47.2 ± 4.3	68.9 ± 3.1	—	4.5
5	16.0 ± 1.6	42.1 ± 3.8	66.8 ± 4.6	—	4.2

Post-treatment of spores irradiated at 100 ergs/mm.² had previously been found to cause no significant effect upon the proportion of variants induced at this dose (Wainwright & Nevill, 1955). Thus, it was possible to compare the

maximal proportion of variants observed after post-treatment of spores irradiated at a dose of 400 ergs/mm.² with the proportion to be expected from the theoretical curve. The maximal proportion of variants after post-treatment either in distilled water or with iodoacetate was considerably higher than that expected from the theoretical model. Indeed, the maximal proportion found after incubation in distilled water roughly corresponded to that expected for

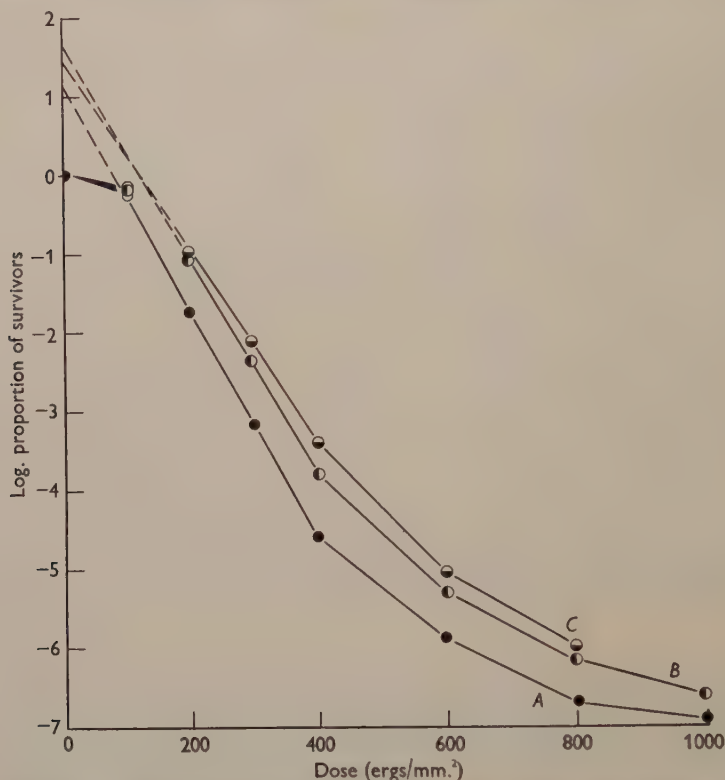


Fig. 5. Influence of post-irradiation treatment upon the dose/survival curve. Irradiated spore suspensions were diluted 4:5 with distilled water and incubated at 37°. Curve *A*: suspensions plated immediately after irradiation and dilution. Curve *B*: suspensions plated after incubation for 24 hr. Curve *C*: suspensions plated after incubation for 96 hr. The logarithm of the proportion of survivors is plotted against dose; the values are corrected for the slight progressive decrease in viable count of the unirradiated control suspension.

a linear dose/mutation curve (Table 2). A detailed study of the effects of post-irradiation incubation was therefore made, and the results of a typical experiment are illustrated in Figs. 4 and 5.

After incubation of irradiated spores in distilled water for 24 hr. the dose/variant-frequency curve (Fig. 4, curve *B*) was of the same form as the curve for spores plated immediately after irradiation (Fig. 4, curve *A*). There was a significant increase in the observed proportion of variants at all doses of radiation exceeding 100 ergs/mm.², but the dose corresponding to the peak of

the curve was unchanged. On incubation for another 24 hr. there was a further increase in the proportion of variants observed with spores irradiated at doses greater than 400 ergs/mm.², but a slight decrease in variant frequency for spores which had received smaller doses of radiation (Fig. 4, curve C). The proportion of induced variants observed for all doses of radiation decreased slightly, but insignificantly, upon further incubation.

The form of the dose/survival curve was unchanged by post-irradiation treatment (Fig. 5), although the extents of survival continued to increase throughout the whole period of the experiment (96 hr.). However, the value of the intercept of the linear portion of the curve increased during the first 24 hr. of treatment, and thereafter tended to decrease again to the value obtained with spores plated immediately after irradiation (Table 3, Expt. 1 corresponds to Figs. 4 and 5). There was considerable variation in the values of these intercepts for different experiments, but the same qualitative changes were observed in all experiments.

Table 3. *Influence of post-treatment on value of the ordinate intercept of dose/survival curves*

Irradiated samples of a spore suspension were diluted 4:5 with distilled water and incubated at 37°. Samples were plated at daily intervals and dose/log. survival curves were prepared from the results obtained. The values of the antilogarithm of the intercept n of the linear portions of the curves are recorded in the table.

Expt. no.	Value of intercept n on ordinate at hr.				
	0	24	48	72	96
1	16	50	35	30	30
2	50	100	100	100	50
3	270	350	300	250	250

DISCUSSION

An increase in survival not accompanied by any significant modification of the proportion of induced variants was observed during the course of post-irradiation treatments with iodoacetate or with distilled water of spores irradiated at all doses up to 1000 ergs/mm.² (Figs. 2, 4 and 5, and Wainwright & Nevill, 1955). A partial resolution of the lethal and mutagenic effects of ultraviolet radiation upon spores of *Streptomyces* sp. strain T 12 has, therefore, been demonstrated over the whole range of radiation doses up to 1000 ergs/mm.².

On the basis of results previously obtained (Wainwright & Nevill, 1955), sets of postulates were given which we believed to represent the minimum requirements for an explanation in terms of lethal and mutagenic 'poisons' of the observed effects of post-radiation treatments upon ultraviolet irradiated spores. The continued increase in survival without modification of the induced variant frequency observed with spores irradiated at doses of radiation exceeding 400 ergs/mm.² (Fig. 2) necessitates an increase in the minimal number of lethal and mutagenic poisons previously postulated. Results obtained in a survey of the effects of post-irradiation treatment with other agents of significance to cell metabolism upon spores of the *Streptomyces*

strain T 12 (in preparation) and upon cells of *Escherichia coli* (Dr Jacobs, personal communication) indicate that yet a further increase in this minimum number of poisons must be postulated.

The effects upon the induced variant frequency of post-irradiation treatment with a given agent have been found to depend upon the dose of radiation used (Fig. 4, and Wainwright & Nevill, 1955), and upon the length of the period of treatment (Figs. 2*b*, 4). Thus, any interpretation of our findings based solely, or partially, upon postulated different effects upon the viabilities of 'provariant' and non-variant spores must be very complex.

Nevertheless, in attempting to make any further analysis of the results obtained in this study, it is important to assess the possible contribution of different effects of the post-treatments upon the viabilities of 'provariant' and non-variant spores which could result in a selectively greater increase in the survival of provariant spores than of the non-variant spores.

For reasons given in detail in the Appendix, we believe that such differences in the effects of the post-treatments upon the viabilities of provariant and non-variant spores are of little, if any, importance in the observed phenomena.

Our results indicate that the increases in variant frequency observed as consequences of post-irradiation treatment cannot be attributed to the associated increases in survival. Hence, it appears that the radiation induces within a fraction of the spores an unstable provariant state which may either revert to the parental non-variant state or be converted to a stable variant condition. Further, the extent to which the provariant spores become stable variants is dependent upon the nature and duration of the post-treatment to which the irradiated spores are subjected. Similar conclusions have been made by Kaplan (1952, 1953) from a study of sectoring in colonies of *Serratia marcescens* (*Chromobacterium prodigiosum*).

Results obtained in a study of the effects of post-irradiation treatment upon the dose/variant-frequency curve differ markedly from results obtained by Hollaender & Emmons (1941) in a similar study with spores of *Trichophyton mentagrophytes* incubated in physiological saline. No explanation of this difference can be given at this time. The results obtained in our own study do not shed any light on the problem of the mechanism of action of the radiation in inducing the provariant state. However, the relationship observed between the maximal proportion of variants found with spores given a dose of 400 ergs/mm.² and the variant frequency for spores irradiated at 100 ergs/mm.² (Table 2) indicates a high degree of reproducibility in the relationship between induction of provariants and radiation dose.

Values of the intercepts at the ordinate of the linear portions of dose/survival curves have been considered to represent the average number of hits or critical events required to inactivate an irradiated cell. Attempts have been made to correlate this value with the average number per cell of some cell component: in particular, the average number of nuclei or sets of chromosomes (e.g. Atwood & Norman, 1949; Norman, 1951; Sarachek & Lucke, 1953). Variations in the values of such intercepts resulting from post-irradiation treatment of non-germinating, uninucleate spores (Fig. 5 and Table 3) clearly impose limits

upon the types of events which can be postulated as leading to cell inactivation, and indicate that killing occurs only as the result of a complex sequence of events.

We are greatly indebted to Dr H. B. Newcombe for criticism and encouragement at all stages of this investigation.

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APPENDIX

The contribution of differential effects upon survival of provariant and non-variant spores in the modifications of induced-variant frequency resulting from post-irradiation treatments

As the period of post-treatment in distilled water of spores irradiated at 400 ergs/mm.² is increased, a stage is reached at which the extent of survival continues to rise but the proportion of variants remains unchanged (Fig. 2*b*). This indicates that if there are any differences in effects which result in a selection favouring provariant spores they progressively decrease during the course of the post-treatment. Our consideration must, therefore, be restricted entirely to effects which may be observed during the early period of treatment of populations of provariant and non-variant spores.

The extent of the increases in variant frequency observed after 3 hr. post-treatment, in distilled water or with iodoacetate (Fig. 1), of spores irradiated at 400 ergs/mm.² were inversely related to the variant frequency observed without post-treatment. The extent of these increases could not be correlated with either the initial level of survival (which varied in different experiments over a 50-fold range) or the extent of the increases in survival. Further, the total concentration of spores was approximately the same in all experiments. Thus, it would appear that the rate of increase in the survival of provariant spores relative to that of non-variant spores would be primarily determined

by the frequency of provariants actually present in the suspension, and would be largely independent of the absolute concentration of viable spores. Hence, as the variant frequency rose during the course of prolonged incubation, the selectively greater increase in the survival of provariant spores than non-variant spores would decrease and become negligible. The variant frequency would, therefore, be expected to rise to a maximal value presumably determined by the initial physiological condition of the spores before irradiation, the nature of the post-treatment applied and perhaps the radiation dose. The maximal variant frequency would not be directly determined by the frequency of variants observed with spores plated without post-treatment, but the time required for the attainment of that maximal value would be determined by the initial variant frequency.

Therefore, if the relative responses of radiation-induced provariant and non-variant spores to a constant selective environment were independent of the radiation dose, it would be expected that the effects of a given post-treatment upon the proportions of variants would (i) be greater upon spore suspensions irradiated at 100 ergs/mm.² than upon those irradiated at 400 ergs/mm.², and (ii) lead to the attainment of approximately the same maximal proportion of variants for all suspensions independently of radiation dose.

Neither prediction is in accord with the results obtained (Fig. 4 and Wainwright & Nevill, 1955). Further, postulates which could be used to explain the discrepancy between prediction and observation at low radiation doses are inadequate to explain the corresponding discrepancy at high radiation doses.

On the other hand, if the relative response of provariant and non-variant spores to a constant selective environment were a function of radiation dose, we would expect a progressive shift of the dose corresponding to the peak of the dose/mutation curve towards a higher dose during the course of the post-radiation treatment. The absolute extent of the shift cannot be predicted as the extent to which the survival of provariant spores would be selectively increased above that of non-variant spores is unknown.

The data presented in Fig. 5 show that the increase in variant frequency resulting from post-radiation incubation in distilled water of spores irradiated at doses exceeding 300 ergs/mm.² is very marked. With spores irradiated at lower doses the increase in variant frequency after similar treatment is smaller, or even negligible. However, there was no apparent change in the dose of radiation corresponding to the peak of the dose/mutation curve. Our data do not preclude the possibility of a small change of this type. Nevertheless, they do indicate that the extent of any such change is smaller than would be expected if the observed increases in variant frequency following incubation in distilled water were due solely, or even largely, to differences in the extents of the increase of survival of provariant and non-variant spores.

It appears, therefore, that differences in the effects of the post-treatments upon the viabilities of provariant and non-variant spores are of little, if any, importance in the phenomena which we have studied.

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Isolation of *Streptomyces* spp. Capable of Decomposing Preparations of Cell Walls from various Micro-organisms and a Comparison of their Lytic Activities with those of certain Actinomycetes and Myxobacteria

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SUMMARY: Dispersion of isolated cell walls of *Streptococcus faecalis* in washed agar provided an opaque medium on which cell-wall decomposing micro-organisms were isolated from soil. All of the organisms isolated on *S. faecalis* cell-wall agar were *Streptomyces* spp. The lytic activities of seven isolates of *Streptomyces*, *S. albus*, two strains of *Micromonospora chaliceae*, *Micromonospora* sp., *Nocardia gardneri* and three strains of *N. corallina*, were studied on cell-wall agar media prepared from five Gram-positive and three Gram-negative bacteria and from the yeast *Candida pulcherrima*. The three strains of *Nocardia corallina* showed no lytic activity on any of the cell-wall substrates. All of the actinomycetes tested were without activity on the Gram-negative cell-wall agar media. Most of the actinomycetes produced lysed zones on Gram-positive cell-wall agar and the greatest lytic activities were observed with *Bacillus megaterium* and *Candida pulcherrima* cell-wall substrates. *Cytophaga johnsonae* and two strains of *Myxococcus fulvus* were lytic on *Candida pulcherrima* cell-wall agar but no lysis occurred on the bacterial cell-wall agars.

Although trypsin lyses certain heat-killed bacteria, in particular those belonging to the Gram-negative group, the cell walls constitute the principal components of the trypsin-resistant residues (Salton, 1953*a*). Thus dispersion of heat-killed bacteria in agar would not provide a suitable substrate for the detection of micro-organisms which produce cell-wall decomposing enzymes; organisms possessing proteolytic enzymes would also exhibit lysed zones due to digestion of protoplasmic constituents of heat-killed cells. With the methods available for the separation of bacterial cell walls from protoplasmic material (Salton & Horne, 1951), isolated cell walls may be used for the preparation of cell-wall agar media. Dispersion of cell walls in washed agar provided an opaque medium suitable for the isolation of cell-wall decomposing micro-organisms from soil; the organisms were detected by the production of lysed zones. This paper describes a method for the isolation of cell-wall decomposing micro-organisms and records the lytic activities of certain actinomycetes and myxobacteria on cell-wall agar media.

METHODS

Preparation of cell walls. Cell walls of the following organisms were prepared: *Streptococcus faecalis* (NCTC 6782); *S. agalactiae* Lancefield group B (NCTC 6175); *Micrococcus lysodeikticus* (NCTC 2665); *Staphylococcus aureus* strain Duncan; *Bacillus megaterium* strain KM; *Escherichia coli* strain B; *Pseudomonas aeruginosa*; *Rhodospirillum rubrum* and *Candida pulcherrima* (*Torulopsis*

pulcherrima). Cells were harvested from liquid media by centrifugation after growth under appropriate conditions. With the exception of *Pseudomonas aeruginosa*, cells were washed twice with distilled water on the centrifuge and suspended in distilled water. *P. aeruginosa* was washed with 1 % saline and suspended in 1 % saline. Cells were disintegrated with Ballotini (Chance Bros. grade 12) beads in the Mickle disintegrator with the exception of those of *Bacillus megaterium* and *Rhodospirillum rubrum* which were disintegrated in the Raytheon 9 Kc. Magnetostriction Oscillator. The subsequent steps in the preparation of cell walls were those outlined by Salton & Horne (1951). The walls of *C. pulcherrima* were deposited from suspension by centrifuging for 15 min. at 3000 r.p.m.

Preparation of cell-wall agar plates. Cell-wall agar plates were prepared as follows: 10 ml. of medium consisting of 1.5 % washed agar, 0.1 % K_2HPO_4 and 0.05 % $MgSO_4 \cdot 7H_2O$ were poured into Petri dishes and allowed to set as a bottom layer; c. 8 ml. melted 2 % washed agar was added to c. 2 ml. concentrated cell-wall suspension (containing 25–35 mg. dry weight cell wall/ml.) and poured as a top layer. Uniformly opaque cell-wall agar plates were obtained and used for the isolation of micro-organisms from soil or for testing the lytic activities of other micro-organisms. As cell walls were not prepared under aseptic conditions, heating cell-wall suspensions for 5 min. at 80° before dispersion in washed agar decreased the number of contaminants appearing on the cell-wall agar, especially when plates were to be incubated for periods of 7 days or more. Preliminary experiments comparing the lytic activities of the various micro-organisms on cell-wall agar prepared from untreated and heated (5 min. at 80°) cell-wall suspensions showed no detectable differences.

Heated-cell agar plates. In some experiments the lytic activities of the micro-organisms on heat-killed cells were studied. The plates were prepared as for the cell-wall agar plates, using instead of cell-wall suspensions, thoroughly washed suspensions of bacteria killed by heating for 5 min. at 100°.

RESULTS

Isolation of cell-wall decomposing micro-organisms from soil

For the isolation of cell-wall decomposing micro-organisms, small grains of soil were placed on the surface of the cell-wall agar, the plates were incubated and examined at intervals for the appearance of lysed zones around the soil grains. Cell-wall agar plates prepared with walls from *Streptococcus faecalis*, *Escherichia coli* or *Rhodospirillum rubrum* were inoculated with soil and incubated at 30°. Lysed zones appeared around the soil grains on *Streptococcus faecalis* cell-wall agar after incubation for several days. No lysed zones were observed even after prolonged incubation of *Escherichia coli* and *Rhodospirillum rubrum* cell-wall agar plates inoculated with soil.

The growth from a number of lysed zones on *Streptococcus faecalis* cell-wall agar was picked off and streaked out on *S. faecalis* cell-wall agar and on glycerol + asparagine agar (Conn, 1921). The latter medium was useful in the purification of the organisms isolated from the cell-wall agar as it enabled the

conservation of cell-wall material for the final testing of purified cultures. Several successive transfers on glycerol + asparagine agar were made and the growth on these plates tested for retention of lytic activity on *S. faecalis* cell-wall agar. All of the single-colony isolations were finally re-tested on *S. faecalis* cell-wall agar, after the organisms had been examined for the presence of contaminants on several different media.

The twelve isolates obtained from the soil-inoculated *Streptococcus faecalis* cell-wall agar plate were identified as *Streptomyces* spp.; seven strains were selected for more detailed studies of morphological and cultural characteristics. A comparison of the characteristics of the seven strains with the descriptions of the seventy-three species of *Streptomyces* given in *Bergey's Manual of Determinative Bacteriology* (1948) does not permit their assignment to any of the described species.

The organisms were maintained on a medium containing 1.5 g. (wet weight) washed, heat-killed *Streptococcus faecalis* cells; 0.1 g. K_2HPO_4 ; 0.05 g. $MgSO_4 \cdot 7H_2O$; 1.5 g. agar/100 ml. medium. All strains showed abundant spore formation on this medium within 48 hr. incubation at 30°, thus providing suitable spore inocula for subsequent experiments.

*A comparison of the lytic activities of Streptomyces spp.
and of some actinomycetes*

To test the lytic activities of the seven strains of *Streptomyces* and a number of related actinomycetes, cell-wall agar was prepared with the walls from a number of bacterial species and from the yeast, *Candida pulcherrima*. Cell-wall agar media were inoculated by streaking some of the growth from the stock cultures of the actinomycetes on the agar surface. The method of testing the various actinomycetes for their abilities to decompose the cell walls is illustrated in Pl. 1, figs. 1 and 2, where the growth and lytic activities of the organisms on *Streptococcus faecalis* cell-wall agar is shown (5 days incubation at 30°).

As shown in Pl. 1, fig. 3, the seven strains of *Streptomyces* isolated on *Streptococcus faecalis* cell-wall agar exhibited greater lytic activities on *Bacillus megaterium* cell-wall agar than that observed on the *Streptococcus faecalis* cell-wall agar. *Nocardia gardneri* and the two strains of *Micromonospora chalceae* also produced more extensive zones of lysis on *Bacillus megaterium* cell-wall agar (shown in Pl. 1, fig. 4) than that observed with *Streptococcus faecalis* cell-wall agar.

The seven strains of *Streptomyces*, *Streptomyces albus*, *Nocardia gardneri*, three strains of *Nocardia corallina*, two strains of *Micromonospora chalceae* and a *Micromonospora* sp. were tested for lytic activity on cell-wall agar media prepared with the walls from five Gram-positive bacteria and from the yeast *Candida pulcherrima*. The three strains of *Nocardia corallina* were devoid of lytic activity on all of the cell-wall agar media; the *Micromonospora* sp. showed no activity or only weak activity on the various cell-wall substrates. When tested on *Staphylococcus aureus* cell-wall agar, *Streptomyces* sp. strain 1B and *S. albus* exhibited weak lytic activity, *Nocardia gardneri* and the two

strains of *Micromonospora chalcone* produced more extensive zones of lysis, and the other actinomycetes were non-lytic. The results of the tests of the lytic activities of the actinomycetes on the cell-wall agar prepared from the other Gram-positive organisms are presented in Table 1.

Table 1. *The lytic activities of some actinomycetes on cell-wall agar media*

Test organism	Plates incubated for 5 days at 30°.				
	<i>S. faecalis</i>	<i>S. agalactiae</i>	<i>B. megaterium</i>	<i>M. lysodeikticus</i>	<i>C. pulcherrima</i>
<i>Streptomyces</i> sp. 1A	++	++	+++	++	++
<i>Streptomyces</i> sp. 1B	++	++	+++	++	++
<i>Streptomyces</i> sp. 2A	+	+	+	+	++
<i>Streptomyces</i> sp. 3-1	++	+	++	++	++
<i>Streptomyces</i> sp. 4A	++	+	++	++	+++
<i>Streptomyces</i> sp. 5A	++	++	+++	++	++
<i>Streptomyces</i> sp. 7-2	++	++	+++	++	++
<i>Streptomyces albus</i>	++	++	+	+	+
<i>Nocardia gardneri</i> NCTC 6531	++	++	+++	++	+++
<i>Micromonospora chalcone</i> (strains SG5 and G7)	++	++	+++	+++	+

+, lytic zone c. 1 mm.; ++, 1-4 mm.; +++, 4+ mm.

None of the actinomycetes showed evidence of lytic activity when tested on cell-wall agar prepared with walls of the Gram-negative organisms *Escherichia coli*, *Pseudomonas aeruginosa* or *Rhodospirillum rubrum*. The absence of lysed zones on *Escherichia coli* cell-wall agar inoculated with actinomycetes is shown in Pl. 1, fig. 5. This is in marked contrast with the extensive lysis observed with most of the actinomycetes (excluding the three non-proteolytic strains of *Nocardia corallina*) on agar containing heat-killed cells of *Escherichia coli* (Pl. 1, fig. 6). Similar results were obtained with heat-killed cells of *Pseudomonas aeruginosa*.

The diffusible nature of the lytic substances (presumably enzymes) produced by the actinomycetes was demonstrated by cutting out blocks of the cell-wall agar from the broad lysed zones and placing them on fresh cell-wall agar. Clearing of the cell-wall agar in the absence of any detectable growth was observed with *Streptococcus faecalis*, *Bacillus megaterium* and *Candida pulcherrima* cell-wall agar blocks.

The lytic activities of several myxobacteria

The chitin-decomposing myxobacterium *Cytophaga johnsonae* studied by Stanier (1947) and two strains of *Myxococcus fulvus* were tested on *Escherichia coli* and *Pseudomonas aeruginosa* cell-wall agar and on heated-cell agar. The two species of myxobacteria produced extensive lysis within several days incubation at 30° on heated-cell agar prepared from the two Gram-negative bacteria. Although *Escherichia coli* and *Pseudomonas aeruginosa* cell-wall agar media supported weak growth of the myxobacteria, there was no evidence of lysis even after 2 weeks incubation at 30°. When tested on *Streptococcus faecalis* and *Bacillus megaterium* cell-wall agar, the myxobacteria showed no lytic activities. However, *Cytophaga johnsonae* and the two strains of *Myxo-*

coccus fulvus had effected extensive lysis on *Candida pulcherrima* cell-wall agar plates incubated for 3 days at 30°; *Cytophaga johnsonae* showed greater lysis than the two strains of *Myxococcus fulvus*.

DISCUSSION

As a group, the actinomycetes possess marked bacteriolytic properties, and the lytic activities of these organisms have been studied extensively by Welsch (1947). It was not surprising that the organisms isolated on bacterial cell-wall agar belonged to the *Streptomyces* group. The bacteriolytic properties of the actinomycetes appear to be due to two groups of enzymes: (1) the proteolytic enzymes responsible for the dissolution of heat-killed bacteria (Tai & van Heyningen, 1951; Muggleton & Webb, 1952; Born, 1952); (2) enzymes capable of bringing about a dissolution of isolated cell walls of certain bacteria, e.g. lysis of group A streptococcus walls by *Streptomyces albus* enzymes (McCarty, 1952*a, b*; Salton, 1952*a*). The ability of *Streptomyces* spp. and other actinomycetes to effect a dissolution of bacterial cell walls is further illustrated in this paper. The cell walls of the lysozyme-sensitive organisms *Micrococcus lyso-deikticus* and *Bacillus megaterium* previously shown to be digested by crystalline egg-white lysozyme (Salton, 1952*b*; 1953*a*) are dissolved by the lytic enzymes produced by various actinomycetes. In addition to the lytic enzymes degrading bacterial cell walls, some of the actinomycetes and myxobacteria lyse the cell walls of the yeast *Candida pulcherrima*.

The method for the isolation of cell-wall decomposing micro-organisms described in this paper has not provided organisms possessing lytic enzymes for Gram-negative bacteria. Attempts to isolate such organisms for *Escherichia coli* from cell-wall enrichment cultures have so far failed. The high lipid contents and the nature of the protein components of the cell walls of Gram-negative bacteria (Salton, 1953*b*) may confer a greater degree of resistance to enzymic digestion than that exhibited by the cell walls of certain Gram-positive bacteria.

This work was commenced during the tenure of a Merck International Fellowship at Prof. R. Y. Stanier's laboratory, University of California, Berkeley; I should like to thank Prof. Stanier for his hospitality and interest and Merck and Co. for the award of a Fellowship. I wish to thank Dr H. L. Jensen for cultures of actinomycetes, Dr McCarty for the strain of *Streptomyces albus*, Dr B. Bachmann for strains of *Myxococcus fulvus* and Mr K. Harvey for photographing the cell-wall agar plates. These investigations were continued during the tenure of a Broodbank Fellowship.

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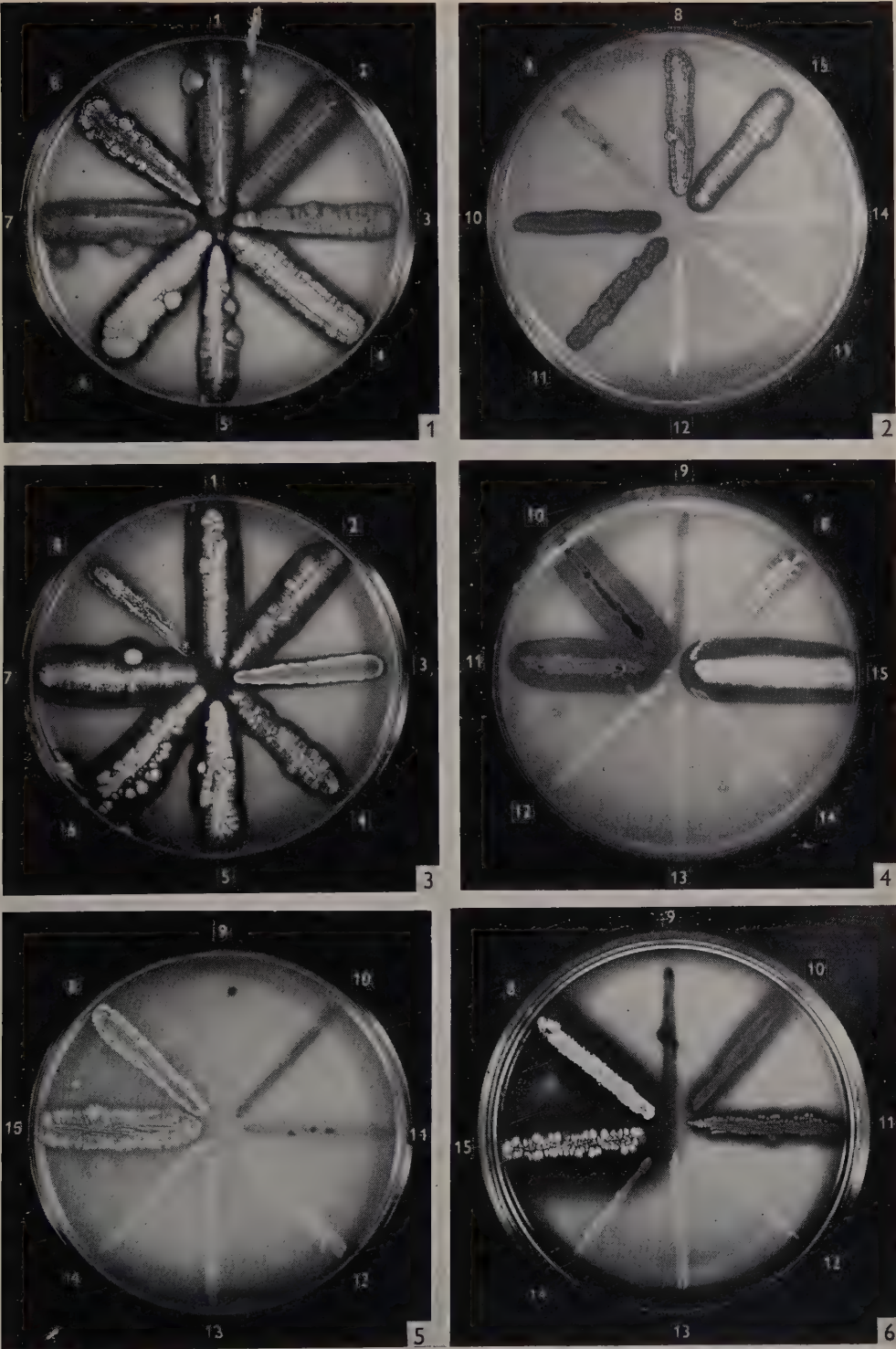
EXPLANATION OF PLATE

- Fig. 1. Lytic activities of the seven strains of *Streptomyces* spp. and *S. albus* on *Streptococcus faecalis* cell-wall agar; 5 days incubation at 30°.
- Fig. 2. Lytic activities of *Micromonospora* spp., *Nocardia* spp. and *Streptomyces albus* on *Streptococcus faecalis* cell-wall agar; 5 days incubation at 30°.
- Fig. 3. Lytic activities of the seven strains of *Streptomyces* and *S. albus* on *Bacillus megaterium* cell-wall agar; 5 days incubation at 30°.
- Fig. 4. Lytic activities of *Micromonospora* spp., *Nocardia* spp. and *Streptomyces albus* on *Bacillus megaterium* cell-wall agar; 5 days incubation at 30°.
- Fig. 5. *Escherichia coli* cell-wall agar inoculated with *Micromonospora* spp., *Nocardia* spp. and *Streptomyces albus*; 7 days incubation at 30°.
- Fig. 6. Lysis of heat-killed *Escherichia coli* by *Micromonospora* spp., *Nocardia gardneri* and *Streptomyces albus* after 3 days incubation at 30°.

Key to test organisms shown in Figs. 1-6:

1	<i>Streptomyces</i> sp.	1A	9	<i>Micromonospora</i> sp.	G1
2	<i>Streptomyces</i> sp.	1B	10	<i>Micromonospora chaliceae</i>	SG 5
3	<i>Streptomyces</i> sp.	2A	11	<i>Micromonospora chaliceae</i>	G7
4	<i>Streptomyces</i> sp.	3.1	12	<i>Nocardia corallina</i>	K6
5	<i>Streptomyces</i> sp.	4A	13	<i>Nocardia corallina</i>	227
6	<i>Streptomyces</i> sp.	5A	14	<i>Nocardia corallina</i>	H2
7	<i>Streptomyces</i> sp.	7.2	15	<i>Nocardia gardneri</i>	NCTC 6531
8	<i>Streptomyces albus</i>				

(Received 24 May 1954)



M. R. J. SALTON—CELL-WALL DECOMPOSING MICRO-ORGANISMS. PLATE 1

CONNER, R. L. & WAGTENDONK, W. J. VAN (1955). *J. gen. Microbiol.* **12**, 31-36.

Steroid Requirements of *Paramecium aurelia*

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SUMMARY: *Paramecium aurelia*, var. 4, stock 51.7 (s) requires a steroid as a growth factor; β - and γ -sitosterol, fucosterol, brassicasterol, stigmasterol and $\Delta^4, 22$ -stigmastadienone are active in supporting its growth. Esterification of the 3-hydroxyl group decreases the activity. The presence of more than one double bond in the ring system inactivates the molecule, as do the more drastic changes of the side chain, as found in diosgenin, digoxigenin, progesterone, estrone and methyl cholate. Oxidation of the ring system destroys activity. The specificity of these requirements suggests that the steroid functions as an essential metabolite for this organism.

Paramecium aurelia, var 4, stock 51.7 (s) has been established in axenic culture in a medium consisting of a yeast extract and proteose peptone, provided a steroid of plant origin is added (van Wagtendonk, Conner, Miller & Rao, 1953). This steroid, identical with either β - or γ -sitosterol or with a mixture of both sterols, was subsequently isolated from lemon juice (Conner, van Wagtendonk & Miller, 1953). The observation that no difference between the growth-promoting activities of β -sitosterol and its optical isomer γ -sitosterol could be detected, led to a study of the molecular configuration of the steroid molecule, necessary for biological activity for *P. aurelia*. A preliminary report has been published (van Wagtendonk & Conner, 1953).

METHODS

Stock cultures of *Paramecium aurelia*, var. 4, stock 51.7 (s) were maintained at 27° in a medium consisting of a yeast extract, proteose peptone and lemon juice. The preparation of the yeast extract, and the preparation of the steroid solutions for assay, as well as the assay technique have been previously described (Conner *et al.* 1953). It should be pointed out here that *P. aurelia* was able to grow at a slow rate in the yeast extract prepared from a few lots of Fleischmann's bakers' yeast (Standard Brands Inc.); in these extracts the active steroids were only stimulatory. The majority of the batches of this yeast yielded extracts in which *P. aurelia* did not grow unless an active steroid was added. Only these batches of yeast extract were used in the survey of the various steroids.

The following steroids were tested in effective concentrations of 800 $\mu\text{g.}$, 400 $\mu\text{g.}$, 200 $\mu\text{g.}$, 100 $\mu\text{g.}$ and 50 $\mu\text{g./ml.}$: cholesterol (U.S.P., Eli Lilly and Co., Indianapolis, Indiana); cholesteryl acetate (Eli Lilly and Co.); epicholesterol, cholestan-3-one, Δ^4 -cholesten-3-one (gifts from Dr C. C. Stock, Sloan Kettering Institute for Cancer Research, and from Dr Helen S. Vishniac,

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Department of Microbiology, Yale University); 3β -cholestanol (three samples respectively from Dr Stock, Dr Vishniac, and from Eli Lilly and Company); 25-keto-norcholestanyl acetate, cerebrosterol, cerebrostenolone, 25-keto-norcholesteryl acetate, coprosterol (Dr H. S. Vishniac); zymosterol (Fleischmann Laboratories through the courtesy of Dr O. N. Breivik); 24-keto cholesterol (Sloan Kettering Institute); 7-keto-cholesteryl acetate (Lederle Laboratories through the courtesy of Dr S. Bernstein); lanost-7-ene-3-ol acetate, lanosta-3-ol acetate, lanost-8-ene-3-ol acetate, lanosta-7, 9(11)-diene-3-ol acetate, lanosta-5, 7-diene-3-ol (gifts of Dr D. H. R. Barton, Birkbeck College, London); ergosterol (Eli Lilly and Co., Dr Barton); $\Delta^{8(9),22}$ -ergostadiene-11-one-3 β -ol-3-acetate, neoergosterol (Eli Lilly and Co.); ergostanyl acetate, β -ergosterol, Δ^7 -ergosterol, γ -dihydroergosterol, ergostanol, ergostane (Lederle Laboratories); ergosterol D acetate, 3β -acetoxy-cervisterol, cervisterol, 3β -acetoxy-5 α -hydroxyergosta-7, 9(11), 22-triene, ergosterol B₃ acetate, 3β -acetoxy-5 α -hydroxyergosta-7, 22-diene, 3β -6 β -diacetoxy-cervisterol, ergosterol B₁, 3β -acetoxy-5 α -hydroxyergosta-8(14)-ene (Dr D. H. R. Barton); brassicasterol, 5-dihydroergosterol (Dr H. S. Vishniac); brassicasteryl acetate, neospongosteryl acetate, chalinasterol, a mixture of stellasterol and stellasterol (Dr W. Bergman, Sterling Chemistry Laboratories, Yale University); β -sitosterol (three samples from Dr H. S. Vishniac, one sample from Dr S. Bernstein); fucosterol (Dr H. S. Vishniac); γ -sitosterol, poriferasterol (Dr W. Bergman); α -spinasterol (one sample from Dr W. Bergman, one sample from Eli Lilly and Co.); stigmasterol, stigmasteryl acetate (Eli Lilly and Co.); stigmastanyl acetate (Lederle Laboratories); α -spinasterol (Dr D. H. R. Barton), $\Delta^{4,22}$ -stigmastadienone (Upjohn Co., through the courtesy of Dr A. C. Ott); chondrillasterol (Merck and Co., through the courtesy of Dr W. J. McAleer); progesterone, estrone, desoxycorticosterone, digoxigenin (Sloan Kettering Institute); methyl cholate, diosgenin (Eli Lilly and Co.); Windaus's keto acid (Dr H. S. Vishniac). The samples were recrystallized several times before use whenever possible. In some cases, however, the amounts available were too small to allow recrystallization, and these compounds were tested as received.

RESULTS

The following steroids and their derivatives were found to be active:

β -sitosterol, elionasterol (γ -sitosterol), fucosterol, brassicasterol, stigmasterol, poriferasterol, brassicasteryl acetate, stigmasteryl acetate and $\Delta^{4,22}$ -stigmastadienone (Figs. 1 and 2). It can be seen from Fig. 1 that the same population density was reached with the two sitosterols and fucosterol. When stigmasterol, poriferasterol or $\Delta^{4,22}$ -stigmastadienone were substituted for the sitosterols a 10 times higher population density was obtained. The response of *Paramecium aurelia* to the addition of brassicasterol was intermediate to that of the sitosterol and the stigmasterol group.

The response of *Paramecium aurelia* to increasing concentrations of stigmasterol and stigmasteryl acetate, and brassicasterol, and its acetate are given in Fig. 2. The acetates, at low concentrations, were less efficient in promoting

the growth of *P. aurelia*. At higher concentrations the growth-promoting activity of stigmasteryl acetate and brassicasteryl acetate approached that of the corresponding free steroids.

None of the other steroids tested showed any growth-promoting activity for *Paramecium aurelia*.

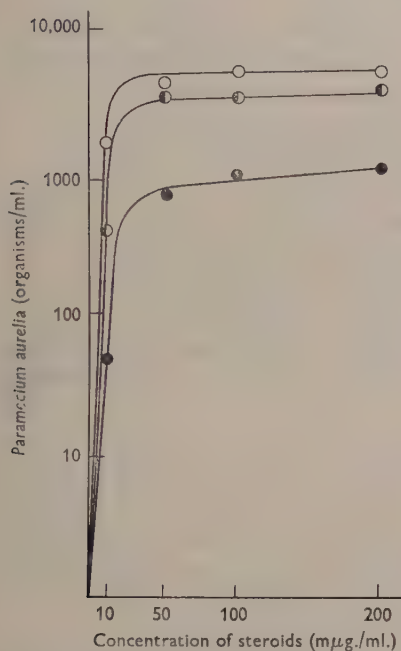


Fig. 1

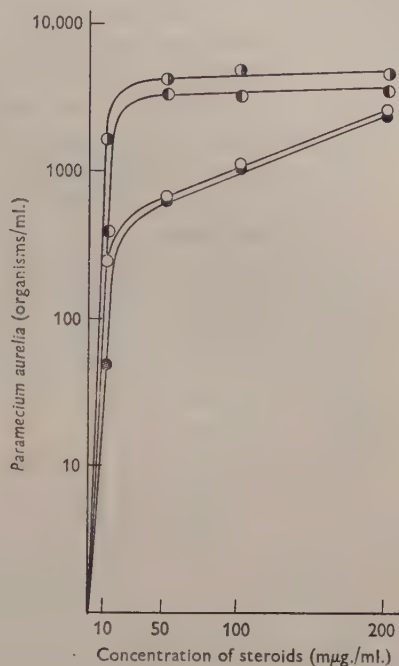


Fig. 2

Fig. 1. Growth response of *Paramecium aurelia*, var. 4, stock 51.7 (s) in axenic culture to increasing concentrations of the sitosterol group (●); brassicasterol (●); stigmasterol, poriferasterol, or $\Delta^{4,22}$ stigmastadienone (○).

Fig. 2. Growth response of *Paramecium aurelia*, var. 4, stock 51.7 (s) in axenic culture to increasing concentrations of: stigmasterol (●); stigmasteryl acetate (●); brassicasterol (●); brassicasteryl acetate (○).

DISCUSSION

A comparison of the structures of active and inactive steroids leads to the following conclusions:

- (1) Steroids having the cholesterol configuration are inactive.
- (2) Addition of either an ethyl- or vinyl group at C₂₄ imparts activity to the molecule, since the sitosterols and fucosterol are active.
- (3) Optical isomerism at C₂₄ does not influence the activity, β - and γ -sitosterol, and stigmasterol and poriferasterol are equally active.
- (4) Unsaturation of the side chain at C_{22,23} enhances the activity; stigmasterol, poriferasterol, and $\Delta^{4,22}$ -stigmastadienone are more active than the sitosterols.

(5) It seems probable that the addition of a methyl group at C₂₄ also imparts activity to the steroid molecule, since brassicasterol is active. It could not be

established whether the addition of the methyl group by itself is sufficient for growth-promoting activity (like the addition of an ethyl group) or whether the addition of a double bond at C_{22,23} is also necessary. The compound which would establish this point, ergosta-5-ene-3 β -ol was not available for testing.

(6) Saturation of all the double bonds destroys activity, as testified by the inactivity of ergosterol and stigmastanyl acetate.

(7) The presence of a second double bond in the ring system inactivates the molecule. Ergosterol, ergosta-8(9), 14, 22-triene-3 β -ol (ergosterol B₁), ergosta-7, 9(11), 22-triene-3 β -ol acetate (ergosterol D acetate), and ergosta-7, 14, 22-triene-3 β -ol acetate (ergosterol B₃ acetate) were all inactive.

(8) Esterification of the 3-hydroxyl group lowers the activity (Fig. 2).

(9) Oxidation of the 3-hydroxyl group to a ketone group and the simultaneous rearrangement of the double bond from C_{5,6} to C_{4,5} does not affect the activity since $\Delta^{4,22}$ -stigmastadienone is as active as stigmasterol.

(10) Degradation of the side chain destroys activity. Progesterone, testosterone, methyl cholate and desoxycorticosterone are all inactive.

(11) Changing the side chain of the steroid molecule to that of a saponin or of a cardiac aglycone results in inactivity of the molecule, since disogenin and digoxigenin are inactive.

(12) The 5:6 double bond appears to be essential for activity, although some doubt exists concerning this point. Ergost-7-ene-3 β -ol (Δ^7 -ergosterol) is inactive, while stigmast-7-ene-3 β -ol (α -spinastanol) is active. It is possible that either or both of these two samples was contaminated. The growth-promoting activity of stigmast-7-ene-3 β -ol may have been due to a contaminant with growth-promoting activity (stigmasterol) or the ergost-7-ene-3 β -ol could have been contaminated with a toxic compound.

At present only a few micro-organisms and one mammalian species are known to require exogenous steroids for growth. The studies of Cailleau (1937, 1938a, b, 1939) indicated a true metabolic requirement for steroids for *Trichomonas columbae*, *T. foetus*, *Eutrichomastix colubrorum* and *Trichomonas batrachorum*. Vishniac & Watson (1953) studied the steroid requirements of the myxothallophyte *Labyrinthula vitellina* var. *pacifica*. The other micro-organisms which require steroids are organisms of the pleuropneumonia group (Edward & Fitzgerald, 1951), and *Peranema trichophorum* (Storm & Hutner, 1953). Ross, van Wagtenonk & Wulzen (1949) and van Wagtenonk & Wulzen (1951) reported that a steroid, later identified as stigmasterol (Kaiser & Wulzen, 1951), functioned as an essential metabolite for guinea-pigs. The specificity requirements for these organisms vary widely, as is evident from Table 1.

It is difficult to draw definite conclusions because of the many gaps in the table. A comparison can be made between the specificity requirements of the two organisms which have been most intensively investigated, *Labyrinthula vitellina* and *Paramecium aurelia*. The similarities between the specificity requirements are the following: (1) the oxidation of the 3-OH group does not affect activity; (2) the presence of more than one double bond in the ring structure inactivates the steroid; (3) degradation of the side chain, or changing

the character of the side chain also inactivates the molecule. The differences between the specificity requirements are striking: (1) the presence of an ethyl group, a vinyl group or a methyl group (?) is essential for activity in the case of *P. aurelia*, while it is not for *Labyrinthula vitellina*; (2) esterification of the 3-OH group lowers the activity of the steroid for *Paramecium aurelia*, while such a treatment completely inactivates the compound for *Labyrinthula vitellina*; (3) unsaturation of the side chain at C_{22,23} enhances the activity of the steroid for *Paramecium aurelia*, while it inactivates the same compound for

Table 1. *A comparison of the growth-promoting activity of various steroids*

+ = active; - = inactive; . = not tested.

Compound	<i>Trichomonas columbae</i>	<i>Labyrinthula vitellina</i>	<i>Peranema trichophorum</i>	<i>Paramecium aurelia</i>	<i>Trichomonas foetus</i> <i>T. batrachorum</i>		Pleuro-pneumonia-like organisms
					<i>Eutrichomastix colubrorum</i>	Guinea-pig	
Cholesterol	+	+	+	-	+	-	+
β -sitosterol	+	+	+	+	.	+	.
γ -sitosterol	.	-	.	+	.	+	.
Fucosterol	.	+	.	+	.	+	.
Stigmasterol	.	-	+	+	.	+	+
Poriferasterol	.	.	+	+	.	.	.
Brassicasterol	.	-	+	+	.	.	.
Ergosterol	+	-	+	-	.	.	.
5-dihydro ergosterol	.	-	.	-	.	.	.
Zymosterol	.	-	.	-	.	.	.

Labyrinthula vitellina. The evidence suggests that the active steroids are not themselves the essential metabolite but precursors for it. This essential metabolite will not be one of the steroids which the cells contain in such great variety and in relatively large quantities, but will be a steroid present in only trace amounts which have hitherto escaped detection. These steroids probably function in a variety of organisms as a cofactor for some essential metabolic process, although it does not necessarily follow that the essential metabolite be the same for each organism tested.

The authors wish to express their gratitude to those (indicated in the text) who so generously donated the steroids used in this investigation. This work was carried out under the auspices of a grant from Eli Lilly and Co., a grant from the National Cancer Institute of the National Institutes of Health (No. C-2160 (C)-), and grants from the Rockefeller Foundation and Indiana University. Partly based on a thesis presented by the senior author in partial fulfilment of the requirements for the Ph.D. degree by the Indiana University. This paper is contribution no. 485 from the Department of Zoology, Indiana University.

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HENDERSON, M. E. K. & FARMER, V. C. (1955). *J. gen. Microbiol.* 12, 37-46.

Utilization by Soil Fungi of *p*-Hydroxybenzaldehyde, Ferulic Acid, Syringaldehyde and Vanillin

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SUMMARY: A number of fungi isolated from soils under a variety of vegetational types was found to attack *p*-hydroxybenzaldehyde, ferulic acid, syringaldehyde and vanillin. These compounds were used as sole source of carbon by the organisms tested. By means of spectrochemical methods and paper chromatography it was shown that vanillin and ferulic acid were converted to vanillic acid before the breaking of the benzene ring, and syringaldehyde was converted to syringic acid. The bearing of these results on the breakdown of lignin in soil is discussed.

This investigation was made in connexion with a study of lignin decomposition by soil fungi. Owing to the difficulty of preparing pure lignin extracts, the work was based on the utilization, as carbon sources, of the aromatic compounds *p*-hydroxybenzaldehyde, ferulic acid, syringaldehyde and vanillin (Fig. 1), which are believed to be related structurally to the lignin molecule, the aldehydes having been obtained on its chemical degradation (Brauns, 1952). The fungi were isolated from soils under a variety of vegetational types.

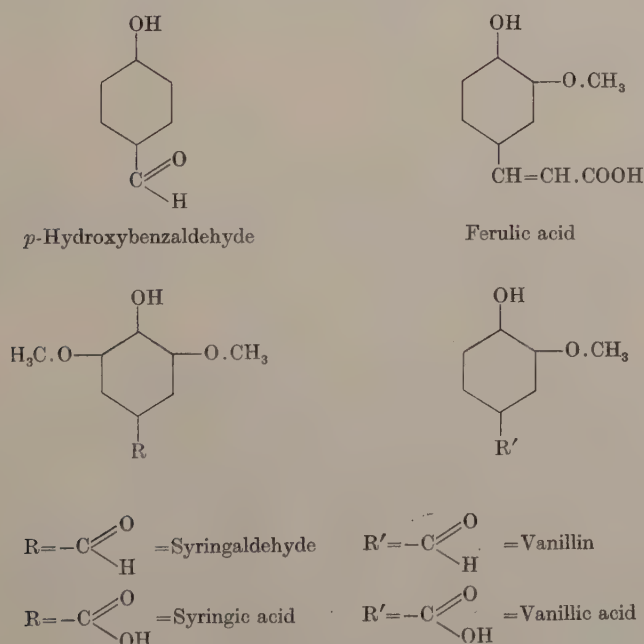


Fig. 1

METHODS

Sampling of soils

The collection of soil samples had to be modified according to the nature of the profile in the various areas sampled. These ranged from open sand to forest. Where possible, a freshly exposed profile about 8 in. deep was sampled by scraping upwards with a sterilized scoop from a depth of $6\frac{1}{2}$ to $\frac{1}{2}$ in. The omission of the surface $\frac{1}{2}$ in. was intended to eliminate aerial contaminants not occurring naturally in the soil. The soil was immediately transferred to sterile jars. Each final sample consisted of soil from three profiles dug at random in the area being investigated. Where forest litter was being collected, the fingers were wiped with alcohol prior to picking up litter from the different layers. The samples were stored in a refrigerator until the following day, when dilutions were made.

Isolation of fungi

Fungi were isolated from the samples by the dilution-plate technique (Brierley, Jewson & Brierley, 1928), the medium being modified Waksman's agar (Waksman, 1922). The modifications were: (a) replacement of peptone by $(\text{NH}_4)_2\text{SO}_4$, to decrease the rate of spread of rapidly growing fungi; (b) addition of tannic acid at a concentration of 0.1 % (w/v). The second modification was based on the work of Bavendamm (1928) and of Davidson, Campbell & Blaisdell (1938) who showed that those wood-rotting fungi which are most active in decreasing the lignin content, i.e. the 'white rots', can be differentiated by their reaction with tannic acid which they oxidize to a brown product. A number of other substances give a similar reaction, but tannic acid has the advantages of being only slightly toxic and of inhibiting the growth of most bacteria (Fahreus, 1949).

The plates were incubated at 21.5° , and it was possible to remove colonies up to the 10th or 12th day of incubation. Representative isolates from the dilution plates were transferred to potato glucose agar for purification and identification. A selected number of isolates from each soil area sampled were retained in stock cultures on potato glucose agar; they included fungi which did not oxidize tannic acid in addition to those which did.

Cultural conditions

The basal medium was modified Czapek's mineral salts; sucrose and FeSO_4 were omitted, the latter because of its reaction with phenolic compounds to form coloured products. The basal medium thus contained: NaNO_3 , 1.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g.; KH_2PO_4 , 0.5 g.; KCl, 0.25 mg. in 500 ml. water. To volumes of this solution were added, as required, *p*-hydroxybenzaldehyde, syringaldehyde or vanillin at final concentrations of 0.01 % (w/v), or ferulic acid at 0.005 % (w/v). The concentrations of the organic compounds in the growth experiments were kept low as they are inhibitory to at least some of the fungi at slightly higher concentrations (0.04 %, w/v).

Sterilization of the basal medium was by autoclaving and of the phenolic

compounds by filtration. The medium was dispensed in 10 ml. lots in 4 in. diameter Petri dishes. The inocula were uniform 5 mm. diameter disks cut from potato glucose agar plates.

Growth after 24 days at 21.5° was compared with that on similarly inoculated mineral salt basal medium as control. Since the phenolic compounds constituted the sole source of carbon and were present in such low concentrations, none of the fungi produced abundant growth. However, in the majority of cases, growth visibly exceeded that on the control and was correlated with the disappearance of the phenolic compounds from the medium.

Evidence for utilization of phenolic compounds as a source of carbon

In the main series of growth experiments, although the aromatic compounds constituted the sole source of carbon added to the media, it might be claimed that sufficient nutrients to support considerable growth were carried over in the inoculation disk of agar. However, evidence in support of the view that the phenolic compounds were being utilized as a source of carbon was obtained in the following manner, using *Coniothyrium* sp. and *Hormodendrum* sp. Inocula cut from the margins of colonies growing in liquid media containing one of the phenolic compounds were used to inoculate fresh dishes of liquid medium. These inocula were cut at a considerable distance from the original inoculation disk and part of the resulting growth was taken for further inoculation. Such successive transfers were continued at monthly intervals for a period of 4 months; the growth in the presence of the various phenolic compounds greatly exceeded that in the mineral salt controls. As small inocula were taken for the successive transfers, very low amounts of nutrients would be carried from one dish to the next and nutrients to support further growth must have been derived from the medium.

Absorption spectrometry

The absorption spectra of the culture media provided a rapid means for surveying the metabolism of a large number of fungi. The disappearance of the originally added phenolic compounds was readily followed, and where the metabolic products had absorption in the ultraviolet region, their nature was often indicated; in the case of vanillic and syringic acids, they were positively identified. The procedure was as follows. After 24 days at 21.5° the culture media were removed from the dishes and restored to their original volumes of 10 ml. They were then diluted 10 times and their absorption spectra measured in both acid ($0.05\text{ N-H}_2\text{SO}_4$) and alkaline (0.05 N-NaOH) solutions, in the range 400 to $240\text{ m}\mu$. The lower wavelength was set by the absorption of the mineral salts in the medium.

In the interpretation of the results, the following situations arose:

(a) No measurable absorption, indicating that no aromatic compounds remained in the culture medium.

(b) Absorption identical with that of the original substrates: their identification and estimation was then certain and accurate.

(c) Absorption of vanillin and ferulic acid completely replaced by that of vanillic acid, and that of syringaldehyde by syringic acid: their identification and estimation were also certain and accurate.

(d) Absorption corresponding to a mixture of (i) vanillin and vanillic acid, (ii) syringaldehyde and syringic acid, or (iii) ferulic and vanillic acids. The identification of the metabolic products was then less certain but reasonable. Care was taken that the whole absorption spectrum in both acid and alkaline solution was consistent with this interpretation.

The absorption maxima of the pairs (i) and (ii) above are well separated in alkaline solution (Lemon, 1947) and so their concentration was readily estimated. It was not possible, however, to identify and estimate with any certainty the presence of small amounts of vanillic acid associated with ferulic acid (absorption maxima 298 and 345 m μ respectively in the alkaline solution).

(e) Absorption appeared which could not be ascribed to the above compounds alone. When present, this absorption was usually of a weak diffuse nature and did not interfere with the estimation of the residual amounts of phenolic compound originally added. The presence of vanillic and syringic acids, however, could not be established with any certainty, although a figure could be given for the maximum concentration likely to be present.

Paper chromatography

Flasks (250 ml.) containing 100 ml. medium were inoculated with five disks cut from potato-glucose agar plates and incubated at 21.5° for periods of 7 and 14 days. The resulting growth was removed by filtration and the filtrate acidified. Thereafter the filtrate was extracted 3 times with 10 ml. ether. The ether was removed by evaporation and the residue dissolved in a few drops of absolute ethanol. The spots were applied to Whatman no. 1 filter-paper by means of a capillary pipette, each extract being spotted 8 times.

The papers were developed with a single-phase mixture based on Long, Quayle & Stedman (1951), consisting of *n*-butanol/ammonia (sp.gr. 0.880)/water (80/5/15 vol.). They were run for 16 hr. at 21° using the descending method. To separate ferulic and vanillic acids it was necessary to run the papers for 28 hr. Duplicate papers were run each time, one being sprayed with 2:4-dinitrophenylhydrazine (Bland, 1949) to trace the aldehydes, the other with diazotized sulphanilic acid (Bray, Thorpe & White, 1950) to reveal the acids and any other phenolic compounds.

RESULTS

Growth experiments

A total of sixty-one isolates, representative of those fungi isolated from ten different soil areas, was studied (Table 1). With the exception of two *Mucor* isolates these were all Fungi Imperfecti. No ascomycetes or basidiomycetes were identified among the organisms isolated, although some may have been present among a number of unidentified non-sporing fungi.

Spectrochemical analyses of the media at the end of the growth experiments

Table 1. *List of organisms (genera) studied and the substrata from which they were isolated*

Numbers in brackets indicate the number of isolates.

Organism	Location
<i>Acrostalagmus</i> (1)	Agricultural soil
<i>Alternaria</i> (1)	Corsican Pine litter
<i>Aspergillus</i> (2)	Foreshore sand; garden soil
<i>Botrytis</i> (2)	Fixed dune sand; heath soil
<i>Cephalosporium</i> (3)	{ Scots Pine humus; peat Foreshore sand
<i>Chaetomella</i> (2)	Ammophila dune sand; fixed dune sand
<i>Coniothyrium</i> (2)	Scots Pine litter; foreshore sand
<i>Cylindrocarpon</i> (1)	Deciduous wood soil
<i>Fusarium</i> (1)	Ammophila dune sand
<i>Gliocladium</i> (1)	Agricultural soil
<i>Haplographium</i> (1)	Deciduous wood soil
<i>Hormiscium</i> (2)	Deciduous wood soil; Ammophila dune sand
<i>Hormodendrum</i> (5)	{ Sand under Scots Pine (afforested sand dune); garden soil; foreshore sand (2); peat
<i>Monilia</i> (1)	Heath soil
<i>Mucor</i> (2)	Foreshore sand; Corsican Pine humus
<i>Papularia</i> (1)	Fixed dune sand
<i>Penicillium</i> (15)	{ Foreshore sand; Ammophila dune sand; Dune heath soil; fixed dune sand (2); Corsican Pine humus; heath sand; Scots Pine humus; peat (3); moor soil (4)
<i>Pullularia</i> (1)	Scots Pine litter
<i>Pyrenochaeta</i> (1)	Ammophila dune sand
<i>Sphaeronaema</i> (1)	Ammophila dune sand
<i>Spicaria</i> (1)	Foreshore sand
<i>Sporotrichum</i> (2)	Heath soil (2)
<i>Tilachlidium</i> (1)	Ammophila dune sand
<i>Torula</i> (2)	Heath soil; pasture soil
<i>Trichoderma</i> (3)	{ Sand under Corsican Pine (afforested sand dune); Corsican Pine humus; deciduous wood soil
<i>Trichosporium</i> (1)	Foreshore sand
<i>Verticillium</i> (2)	Garden soil; foreshore sand
Unidentified (3)	Scots Pine litter; peat; Ammophila dune sand

revealed that the majority of the fungi decomposed these aromatic compounds to a certain extent. The amounts of substrate remaining after a 24-day period of incubation are listed in Table 2.

Examination of this table shows that the most active organisms were *Alternaria* sp., *Aspergillus* sp., *Chaetomella* sp., *Coniothyrium* sp., *Cylindrocarpon* sp., *Hormiscium* spp., *Hormodendrum* spp., *Penicillium* sp. (no. 8), *Pyrenochaeta* sp., *Sphaeronaema* sp. and *Torula* spp., which caused complete disappearance of the benzene ring of all four compounds during the course of the experiment. It can also be seen that some fungi attacked the different compounds to a different extent. *p*-Hydroxybenzaldehyde was the most readily attacked, being removed by all organisms with the exception of unidentified organism no. 3 and a *Mucor* sp. Syringaldehyde was not as readily available as vanillin, a number of fungi completely altering vanillin, but leaving considerable quantities of syringaldehyde, namely *Aspergillus* sp. (no. 2), *Botrytis* sp. (no. 2), *Cephalosporium* spp. (nos. 1 and 3), *Haplographium* sp.,

Table 2. *Residual amounts of phenolic compounds added initially, and amounts of products from them, after growth of various fungi for 21 days at 21.5° on mineral salt basal medium + given carbon compound*

p-Hydroxybenzaldehyde (0.01%, w/v, initially) was completely removed by all organisms tested except (i) *Botrytis* sp. no. 1 gave an unidentified product with detectable absorption; (ii) *Mucor* sp. no. 2 gave 0.0043% and unidentified organism no. 3 0.0047% residual *p*-hydroxybenzaldehyde.

	Phenolic compound (% w/v) added initially					
	Ferulic acid (0.005)		Syringaldehyde (0.01)		Vanillin (0.01)	
	Amount of residue of initial substance or of product after growth (% w/v)					
	Ferulic acid	Vanillic acid	Syring- aldehyde	Syringic acid	Vanillin	Vanillic acid
<i>Acrostalagmus</i>	—	—	—	< 0.0010	—	—
<i>Alternaria</i>	—	—	—	—	—	—
<i>Aspergillus</i> no. 1	—	—	—	—*	—	—
<i>Aspergillus</i> no. 2	—	U*	0.0043	0.0047	—	—
<i>Botrytis</i> no. 1	—	U*	0.001	U	0.0013	< 0.002
<i>Botrytis</i> no. 2	< 0.001	U*	0.0051	U	—	< 0.0010
<i>Cephalosporium</i> no. 1	0.0052	Indicated*	0.0018	0.006	—	0.011
<i>Cephalosporium</i> no. 2	0.0030	0.0025	—	0.010	—	0.0095
<i>Cephalosporium</i> no. 3	—	—	0.0048	0.0060	—	—
<i>Chaetomella</i> no. 1	—	—	—	U*	—	—
<i>Chaetomella</i> no. 2	—	—	—	—	—	—
<i>Coniothyrium</i> no. 1	—	< 0.0010	—	U*	—	—
<i>Coniothyrium</i> no. 2	—	—	—	—	—	—
<i>Cylindrocarpon</i>	—	—	—	—	—	—
<i>Fusarium</i>	—	—	0.002	0.0023	—	—
<i>Gliocladium</i>	—	—	—	0.006*	—	—
<i>Haplographium</i>	—	—	0.0065	—	—	—
<i>Hormiscium</i> no. 1	—	—	—	—	—	—
<i>Hormiscium</i> no. 2	—	—	—	—	—	—
<i>Hormodendrum</i> no. 1	—	—	—	—	—	—
<i>Hormodendrum</i> no. 2	—	—	—	—	—	—
<i>Hormodendrum</i> no. 3	—	—	—	—	—	—
<i>Hormodendrum</i> no. 4	—	—	—	—	—	—
<i>Hormodendrum</i> no. 5	—	—	—	—	—	—
<i>Monilia</i>	—	0.0023*	0.0090	—	0.0081	0.0010
<i>Mucor</i> no. 1	0.0055	—	0.0066	< 0.0010	0.0036	U
<i>Mucor</i> no. 2	U*	U*	0.0022	0.0020	0.0025	U*
<i>Papularia</i>	< 0.0005	U*	—	< 0.001	—	—
<i>Penicillium</i> no. 1	—	—	0.0053	0.0041	—	—
<i>Penicillium</i> no. 2	—	—	0.0045	0.0025	—	—
<i>Penicillium</i> no. 3	—	—	0.0042	0.0070	—	U*
<i>Penicillium</i> no. 4	—	—	0.0011	0.0085	—	—
<i>Penicillium</i> no. 5	—	—	0.0027	0.0035	—	—
<i>Penicillium</i> no. 6	—	—	0.0029	0.0060	—	—
<i>Penicillium</i> no. 7	—	—	—	0.0100	—	—
<i>Penicillium</i> no. 8	—	—	—	—	—	—
<i>Penicillium</i> no. 9	—	—	0.0073	0.0020	—	—
<i>Penicillium</i> no. 10	—	—	—	0.0075	—	—
<i>Penicillium</i> no. 11	—	—	0.0007	0.0095	—	—
<i>Penicillium</i> no. 12	—	—	—	0.0031	—	—
<i>Penicillium</i> no. 13	—	U*	0.0061	0.0050	—	0.001*
<i>Penicillium</i> no. 14	—	—	—	0.0055	—	—

Table 2 (cont.)

	Phenolic compound (% _v w/v) added initially					
	Ferulic acid (0.005)		Syringaldehyde (0.01)		Vanillin (0.01)	
	Amount of residue of initial substance or of product after growth (% _v w/v)					
	Ferulic acid	Vanillic acid	Syring- aldehyde	Syringic acid	Vanillin	Vanillic acid
<i>Penicillium</i> no. 15	—	—	—	0.0063	—	—
<i>Pullularia</i>	<0.001	U*	—	U	<0.0005	U
<i>Pyrenochaeta</i>	—	—	—	—	—	—
<i>Sphaeronaema</i>	—	—	—	—	—	—
<i>Spicaria</i>	—	<0.0015	0.001	0.0015	—	0.007
<i>Sporotrichum</i> no. 1	—	<0.0010	—	0.0083	—	<0.0010
<i>Sporotrichum</i> no. 2	—	<0.0015*	0.0029	0.0073	—	0.0100
<i>Tilachlidium</i>	—	<0.001*	—	0.0076	—	U*
<i>Torula</i> no. 1	—	—	—	—	—	—
<i>Torula</i> no. 2	—	—	—	—	—	—
<i>Trichoderma</i> no. 1	0.0038	Indicated*	0.0072	—	—	0.0085
<i>Trichoderma</i> no. 2	0.0042	—	0.0020	0.0071	—	0.0060
<i>Trichoderma</i> no. 3	0.0044	0.002	0.0062	<0.0010	—	0.0095
<i>Trichosporium</i>	—	<0.001*	—	—	—	—
<i>Verticillium</i> no. 1	0.0055	—	0.0051	0.0045	—	0.0090
<i>Verticillium</i> no. 2	0.0040	Indicated*	0.0010	U*	0.0012	U*
Unidentified no. 1	—	—	0.0033	0.0015	—	—
Unidentified no. 2	—	0.0040*	—	0.0068	—	0.0085
Unidentified no. 3	<0.001	U*	—	U	<0.001	U

*=Identified by paper chromatography; —=no trace; U=unidentified absorption.

the majority of *Penicillium* spp., *Sporotrichum* sp. (no. 2), *Trichoderma* spp. (nos. 1–3) and *Verticillium* sp. (no. 1). Generally, where vanillin was broken down, ferulic acid was also attacked.

Intermediates formed during the breakdown of ferulic acid, syringaldehyde and vanillin

One intermediate product was traced from each of ferulic acid, vanillin and syringaldehyde; ferulic acid and vanillin were converted to vanillic acid, and the syringaldehyde to syringic acid. No intermediate was traced from *p*-hydroxybenzaldehyde. The ability of the fungi to metabolize the acids further varied considerably, as can be seen from the figures in Table 2 showing the amounts of the acids present after the 24-day growth period. *Cephalosporium* spp., *Sporotrichum* sp., *Trichoderma* spp., *Verticillium* sp. and unidentified organism no. 2 showed almost quantitative conversion of vanillin to vanillic acid, at which stage metabolism apparently ceased. *Spicaria* sp. and *Trichoderma* sp. gave lower residual amounts of vanillic acid, indicating an ability to utilize it at a slow rate. Other fungi, including *Hormodendrum* sp. (no. 1) and *Torula* sp. (no. 1) left no residue when grown on vanillin. These two fungi were equally effective in removing the substrate when grown on mineral salts + 0.01 % (w/v) vanillic acid, whereas *Spicaria* sp., *Trichoderma* sp. and unidentified organism no. 2 left considerable quantities of the acids. Syringic

acid was less readily metabolized, a large number of fungi (in particular *Penicillium* spp. which removed vanillic acid completely) leaving considerable quantities of syringic acid in the media.

Further analyses were made with *Hormodendrum* sp. (no. 1) to discover whether it was in fact releasing vanillic and syringic acids as intermediate products in the early stages of growth. Flasks containing 100 ml. of medium were inoculated with five disks and 5 ml. samples were removed aseptically after different lengths of time for spectrochemical examination. Vanillic acid was identified in the vanillin and ferulic acid cultures, and syringic acid in the syringaldehyde culture. The amounts of the residual substrates and the amounts of vanillic and syringic acids formed are recorded in Table 3; these results were confirmed by paper chromatography. The production of vanillic and syringic acids as intermediate products by the following fungi was also confirmed by paper chromatography: *Chaetomella* sp. (no. 1), *Hormiscium* sp. (no. 1), *Penicillium* sp. (no. 3), *Pyrenochaeta* sp. and *Torula* sp. (no. 1). As these fungi are all active in removing the substrates it was necessary to make the chromatographic analyses of the media after a very short growth period.

Table 3. *The amounts of the original substrates remaining and of the intermediates formed after Hormodendrum sp. had been grown on mineral salts with ferulic acid, syringaldehyde or vanillin at 21.5°.*

Samples (5 ml.) were removed aseptically for analysis after the stated number of days.

Period of growth (days)	Compound added as carbon source					
	Ferulic acid		Syringaldehyde		Vanillin	
	Ferulic→Vanillic acid		Syringaldehyde→Syringic acid		Vanillin→Vanillic acid	
	Concentration (% w/v) of original substance or product					
0	0.005	—	0.01	—	0.01	—
3	0.0018	trace*	No analysis		No analysis	
6	trace	—	0.0048	trace	0.0028	trace
8	No analysis		0.0019	0.005*	0.002	0.0025*
14	—	—	—	0.002	—	—

* = Identified by paper chromatography; — = no trace.

A number of fungi formed unidentified metabolic products which interfered with the identification of syringic and vanillic acids by absorption spectrometry. The presence of such unidentified absorption is indicated by the letter U in Table 2. In these instances the culture filtrates were examined by paper chromatography. The results are shown in Table 2 where an asterisk indicates that the presence of the acids was in fact established in most cases.

Two exceptions were *Pullularia* sp. and unidentified organism no. 3, neither of which produced syringic acid from syringaldehyde or vanillic acid from vanillin. It would seem that these fungi attack the compounds in a different manner, indicated by the fact that when grown on syringaldehyde they both colour the solution yellow and on vanillin colour it orange, unidentified

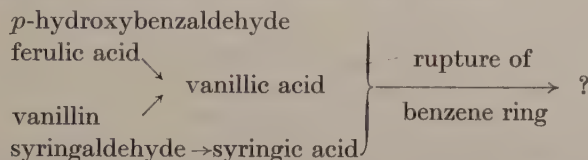
organism no. 3 on vanillin forming a fine orange precipitate. When grown on ferulic acid they both gave a fine orange precipitate but the presence of very small quantities of vanillic acid was shown by paper chromatography.

Two further exceptions were *Botrytis* sp. (no. 1) and *Trichoderma* sp. (no. 1) which instead of forming syringic acid when grown on syringaldehyde gave rise to a substance of low R_F value which was detected by diazotized sulphanilic acid.

DISCUSSION

The data show that there exist in soils under a wide range of vegetational types fungi which can decompose *p*-hydroxybenzaldehyde, ferulic acid, syringaldehyde and vanillin. The fungi studied are representative of those which can be isolated by the dilution-plate technique; not isolated by this technique are the soil basidiomycetes and ascomycetes which should also be studied in order to obtain a true picture of fungal activity in the soil.

The methods of analysis employed, absorption spectrometry and paper chromatography, have shown that the decomposition of these compounds (see Fig. 1) in the majority of cases proceeds as follows:



Absorption within the spectral range used does not permit the identification of the products formed following rupture of the benzene ring. Some fungi formed products (in addition to the acids) which appeared on the paper chromatogram, but they have not yet been identified.

As the original soil isolations were based on the ability of the fungi to oxidize tannic acid, it is interesting to compare this property with their activity in the utilization of these compounds. The results showed that of sixty-one fungal isolates studied, thirty-four oxidized tannic acid, and of these fifteen removed the four phenolic compounds completely from the media. Of the twenty-seven isolates which did not oxidize tannic acid, two removed the compounds completely. Within these two main groups the numbers of fungi which removed three, two, one or none of the compounds were similar. There would thus seem to be a correlation only between the ability to oxidize tannic acid and the ability to utilize all four of the phenolic compounds tested.

Only a few fungi formed coloured products and precipitates such as are produced by certain wood-rot fungi when grown on some of these compounds. Dion (1952) obtained similar products when he added culture filtrate containing an extracellular enzyme of *Polyporus versicolor* to solutions of a number of phenols. As tannic acid oxidation depends on the formation of a quinone and as these soil fungi break down the aromatic ring, it seems probable that an enzyme system is involved which is different from the polyphenoloxidase type present in wood-rot fungi and that the correlation between the number of isolates which utilized all four phenolic compounds and the number which

oxidized tannic acid is probably not significant. Fåhreus (1949) reported oxidation of tannic acid by a soil fungus which was a weak lignin-decomposer and not a true rot fungus. Ledingham & Adams (1942) found that fungi giving similar reactions with tannic acid varied widely in their ability to decompose lignin in the form of calcium ligno-sulphonate. The ability of soil fungi to utilize the four phenolic compounds tested may constitute a stage in the decomposition of lignin. It has been shown (Henderson & Farmer, unpublished) that certain wood-rot fungi will release vanillin and vanillic acid from spruce sawdust. It would seem that the combined activities of these different fungi may be concerned in the breakdown of lignin under natural conditions.

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A Cytological Study of *Caryophanon latum*

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SUMMARY: In addition to structures already known, the trichomes of *Caryophanon latum* are shown to contain metabolically active cytoplasmic granules which agree with the definition proposed for bacterial mitochondria. Evidence is presented for some, at least quantitative, cytochemical differentiation among these granules.

The purpose of this study is to characterize the cytoplasmic structures found in *Caryophanon latum*. This micro-organism was isolated in 1937 and described in 1940 by Peshkoff. It was originally believed to consist of a cylindrical body, containing a varying number of hoop-shaped nuclei and a common protoplasm. Volutin, which sometimes adhered to the 'nuclei', was also described. The 'nuclei' were described as dividing by longitudinal splitting (Peshkoff, 1940, 1946). Pringsheim & Robinow (1947) presented evidence that the organism does not have a coenocytic structure, but rather is made up of discoid cells. This evidence was provided by cell-wall staining and the ability of the organism to break up into its component protoplasts when it was placed in a hypertonic solution of potassium nitrate or sodium chloride. Furthermore, it was shown that Peshkoff's 'nuclei' were intercellular septa. Bisset & Hale (1953), employing a phosphomolybdic acid mordant and methyl green stain, also demonstrated that the trichome consists of many disk-shaped cells. Tuffery (1953) stated that *C. latum* is a multicellular micro-organism with septa, containing a true cell-wall element, which subdivides it into discoid cells. DeLamater (1952) described a mitotic cycle in *Caryophanon*. According to Bisset (1953*a*), the DeLamater nuclear configurations are either artefacts or portions of cell wall. Bisset's criticisms have been answered (DeLamater, 1954).

The organism can be described as a large peritrichously flagellated Gram-negative bacillus, arranged in trichomes. Each trichome consists of a varying number of discoid cells. When observed with the phase microscope, the arrangement of the cells gives the appearance of light and dark striae; numerous highly refractile bodies are present in the dark bands.

MATERIALS AND METHODS

The organism used throughout this study was strain 4A received through the courtesy of Paul S. May, Department of Bacteriology, Philadelphia College of Pharmacy and Science, Philadelphia, Pa. Stock cultures were maintained in 10 % cow-dung extract at room temperature; transfers were made periodically every month. The initial transplant was on a 10 % cow-dung agar slant; after incubating at room temperature for 18 hr., the organisms were suspended in

sterile distilled water and spread on a 10 % cow-dung agar plate. The inoculated plates were incubated for 18 hr. at room temperature. When the medium described by Pringsheim & Robinow (1947) was substituted for the cow-dung agar, the organism grew in pairs instead of in trichomes.

Cow-dung extract was prepared by suspending fresh cow dung in 9 times its own weight of tap water. After mixing, the suspension was allowed to settle for several minutes and the supernatant fluid filtered through cheese cloth. Cow-dung agar was prepared by adding 2 g. of plain agar to each 100 ml. of extract. Both the extract and agar were autoclaved for 30 min. at 15 lb.

The staining procedures were performed in the following manner: a block of agar with growth was cut from a plate and inverted on a coverslip. The impression smears were processed according to any of the following methods.

Cell wall stains. (1) Bisset & Hale (1953), 1 % chloroform extracted methyl green was used in place of ordinary methyl green; (2) Webb's (1954) method.

Metachromatic staining. (1) Neisser stain. (2) Air dried smears were stained with 0.25 % crystal violet for 1 min., washed with water, treated either with 5 % citric acid for 1 min. or 1 % sulphuric acid for 10 sec., washed with water and mounted in Farrant's medium (Arthur H. Thomas Co., Philadelphia, Pennsylvania).

Metaphosphate stain. Ebel (1952).

Polysaccharide stain. Alcian Blue (Arnold Hoffman Co., Providence, Rhode Island; McKinney, 1953).

Lipid stains. (1) Citric acid-Sudan Black B (Davis, Winterscheid, Hartman & Mudd, 1953); (2) Burdon (1946).

Phospholipid stain. Menschik (1953).

Reduction of tetrazoles. Neotetrazolium, blue tetrazolium, and 2, 3, 5-triphenyltetrazolium (General Biochemicals Inc., Chagrin Falls, Ohio). Agar blocks containing organisms were incubated under partially anaerobic conditions at room temperature for 4 hr. in the presence of a 0.01 % aqueous solution of the respective indicator.

Oxidation of Nadi reagent (Hawk, Oser & Summerson, 1947). Agar blocks containing growth were incubated in a solution consisting of 4 ml. distilled water, 0.25 ml. of 1 % aqueous dimethyl-*p*-phenylenediamine and 0.25 ml. of 1 % ethanolic α -naphthol.

Reduction of Janus green B. The observation of the sequence of colour changes was facilitated by inverting a moist coverslip impression smear on a wet piece of filter-paper, to which was added approximately 0.1 ml. of 0.012 % aqueous Janus green B (Coleman & Bell Co., Norwood, Ohio).

Nuclear stain. DeLamater (1951); best results were obtained when the preparations were hydrolysed for 5 min. in 1 N-HCl at 60° and stained for 12 hr. in a mixture of 10 ml. 0.25 % aqueous Azure A (Azure A Blend, Harleco, Arthur H. Thomas Co., Philadelphia, Pennsylvania) and 0.01 ml. thionyl chloride.

Light microscopy and micrographs. A Bausch and Lomb research microscope CCTB, with balcoated lenses, was employed. Micrographs were taken on Kodak Microfile film with a lensless Argus C-3 camera mounted on a swivel arm support.

Electron micrograph preparations. Impression smears were made on either collodion membranes which were floated off on water from agar blocks, or 'formvar' (Schawinigan Resins, New York, N.Y.) membranes which were moulded on glass slides and floated off on water. The membranes were supported on 200-mesh copper screen. The instrument used was an RCA EMU 2A model.

RESULTS

The trichome, when observed with the conventional microscope employing oblique illumination, appeared to move with a spiral motion. The cross-walls delimiting the individual cells were barely discernible. A comparatively large eccentric spherical body was usually found in each cell, on occasion two such bodies were present. Several smaller bodies appeared along the inner periphery of the cell wall (Pl. 1, fig. 1).

Cell wall and septa. The Hale method stained the cell wall and cross-walls a vivid green while the cytoplasm was very faintly coloured (Pl. 1, fig. 2). The Webb method stained the cross-walls and cell walls bright red and the cytoplasm a distinct pink (Pl. 1, fig. 3).

Metachromatic granules. The McKinney stain for polysaccharides not only stained the cell wall and cross-walls a bluish green, but also polar bodies which usually were adjacent to the cross-walls (Pl. 1, fig. 4). These spherical bodies when stained with either Neisser's stain or crystal violet-citric acid demonstrated metachromasy. If, however, the impression smears were pretreated with hot water (80°) these bodies no longer appeared, although the cross-walls and cell walls retained their staining properties. Similar results were obtained if the organism was stained with crystal violet-citric acid for metachromasy, followed by the Hale cell-wall stain (Pl. 1, fig. 5).

Metaphosphate deposits. Organisms stained according to the Ebel (1952) method contained coloured granules, some stained more intensely than others (Pl. 1, fig. 6). Preparations pretreated with 80° water for 10 min. did not stain.

Lipid material. The organism contained a great deal of sudanophilic material as was demonstrated by the technique of Davis *et al.* (1953). Negative results were obtained with the Burdon method for free lipid. Areas of greater staining affinity appeared to be localized between narrow band-like areas which stained less intensely (Pl. 1, fig. 7). Organisms pretreated with hot pyridine (60°) for 60 min. were not stained, although hot-water controls were not affected. When the organisms were pretreated with acetone for 1–8 hr. at room temperature, most of the lipids except phospholipids should have been removed; such cells when stained with Sudan black B + citric acid presented numerous sudanophilic areas which corresponded in shape and position to the intensely stained areas noted in fig. 7 (Pl. 1, fig. 8). The Menschik stain for phospholipids showed defined spherical bodies which appeared adjacent to the cell wall (Pl. 1, fig. 9).

Areas of oxidative-reductive properties. The reduction of neotetrazolium resulted in the formation of small discrete reddish purple spherical granules, which were usually located along the periphery of the cell wall, occasionally along the cross-walls (Pl. 1, fig. 10). Blue tetrazolium reduction resulted in the

formation of fewer bluish purple granules of the same size and location as those obtained with neotetrazolium (Pl. 1, fig. 11). Triphenyltetrazolium reduction resulted in the formation of larger red granules which appeared along the periphery of the cell wall as well as adjacent to the cross-walls (Pl. 1, fig. 12).

Oxidation of Nadi reagent, after 3 hr. incubation, resulted in the appearance of peripherally located intense red granules (Pl. 1, fig. 13). The presence of 0.01 M-NaCN in the Nadi reagent prevented the development of such granules.

When Janus green B was added to the filter-paper, the organisms immediately stained homogeneously light blue-green; what appeared to be cell wall and cross-walls stained somewhat more intensely. In about 5 min. the cytoplasmic coloration began to fade and the cell wall and cross-walls also appeared to be stained less intensely. Blue-green spherical bodies appeared adjacent to the cross-walls. Thirty to 40 min. later, these spherical bodies turned red (Pl. 1, fig. 14). Three hours later the bodies were colourless, although the cross-walls and cell wall retained their light blue-green colour. A dilute solution (1: 10 of Neisser's methylene blue, when added to the filter-paper after the cells rendered the Janus green B colourless, stained the same spherical bodies purple (Pl. 1, fig. 15). The location of the red granules was verified by removing an impression smear from the filter-paper while the bodies were red, and staining for cell wall with the Hale method. The majority of the cells retained their red bodies; these appeared adjacent to the septa (Pl. 1, fig. 16).

Nuclear structures. The nuclei assumed different shapes and sites in relation to the cross-walls and cell wall in different cells. In some cells they appeared as spherical bodies in close proximity to the cross-walls, in others they appeared as ellipsoidal structures adjacent to them; however, most frequently they appeared as 'Chinese characters' equidistant to the cross-walls and cell walls (Pl. 1, fig. 17).

Electron microscopic observations. The organisms were homogeneously electron-opaque (Pl. 2, fig. 18). Flagella-like structures appeared to be folded along the scalloped sides (these are not shown in the illustrations). When the organism was exposed to high intensity electron beams, 'blow outs' developed. These volatilized areas varied in number, size and location in different cells. The cytoplasm remained dense (Pl. 2, fig. 19). It has previously been demonstrated (Hillier, Mudd, Smith & Beutner, 1950) that electron irradiation 'fixes' bacterial protoplasm. Therefore, a different field on the same preparation was located by scanning with a weak electron beam, and subsequently exposing the organism to an intense electron bombardment by increasing the intensity of the electron beam. A similar, but more violent explosive phenomenon was observed. The cytoplasm was noticeably less opaque, and the cross-walls were clearly distinguishable (Pl. 2, fig. 20). The exploded areas did not appear if the impression smear was pretreated with 80° water for 10 min.

DISCUSSION

The trichomes of *Caryophanon latum* are known to consist of discoid, nucleated cells separated by intercellular septa. With these well-documented observations our own are in accord. Also Pringsheim & Robinow (1947) have reported

that: 'In addition to the nuclear structures the cytoplasm usually contains varying numbers of small metachromatic granules which are conspicuous in fixed and stained cells not pretreated with HCl, but absent from the cytoplasm of hydrolysed bacteria.' Frequent association of 'volutin' and of metachromatic granules with what are now recognized as the intercellular septa was noted respectively by Peshkoff (1940) and by Pringsheim & Robinow (1947). These observations we also confirm and extend, with the observations that the volutin or metachromatic granules are dissolved by hot water and may be volatilized by the electron beam (cf. König & Winkler, 1948; Mudd, Winterscheid, De Lamater & Henderson, 1951; Ruska, Bringmann, Neckel & Schuster, 1952). In addition, we demonstrate characteristic mitochondrial oxidative-reductive activities of cytoplasmic granules with respect to the Nadi reagent, tetrazoles and Janus green B.

We are particularly interested in whether or not cytochemical differentiation among the several cytoplasmic granules may be demonstrated. In the mycobacteria examined by Mudd, Winterscheid *et al.* (1951) and by Winterscheid & Mudd (1953), the cytoplasmic granules showed redox activities with all the indicators, and stained metachromatically with alkaline methylene blue; functional differentiation among the granules was not detected. In *Salmonella typhosa* and *Corynebacterium diphtheriae*, on the other hand, cytochemical differentiation among the cytoplasmic granules was detected (Davis *et al.* 1953; Davis & Mudd, 1955).

The present observations on *Caryophanon latum* suggest at least quantitative, and possibly qualitative, cytochemical differentiation among the cytoplasmic granules. The granules whose position is along the axis of the trichome and usually adjacent to the intercellular septa exhibit strong metachromatic staining and give the characteristic range of colours with Janus green B. The granules, usually smaller, which occur along the cylindrical side-walls reduce the tetrazoles and oxidize the Nadi reagent, but are at least not readily demonstrable by Janus green B or acidified basic (metachromatic) dyes. Both types of granule colour with Ebel's metaphosphate stain and with the citric acid-sudan black B stain for phospholipid, and both appear to be volatilizable by the electron beam.

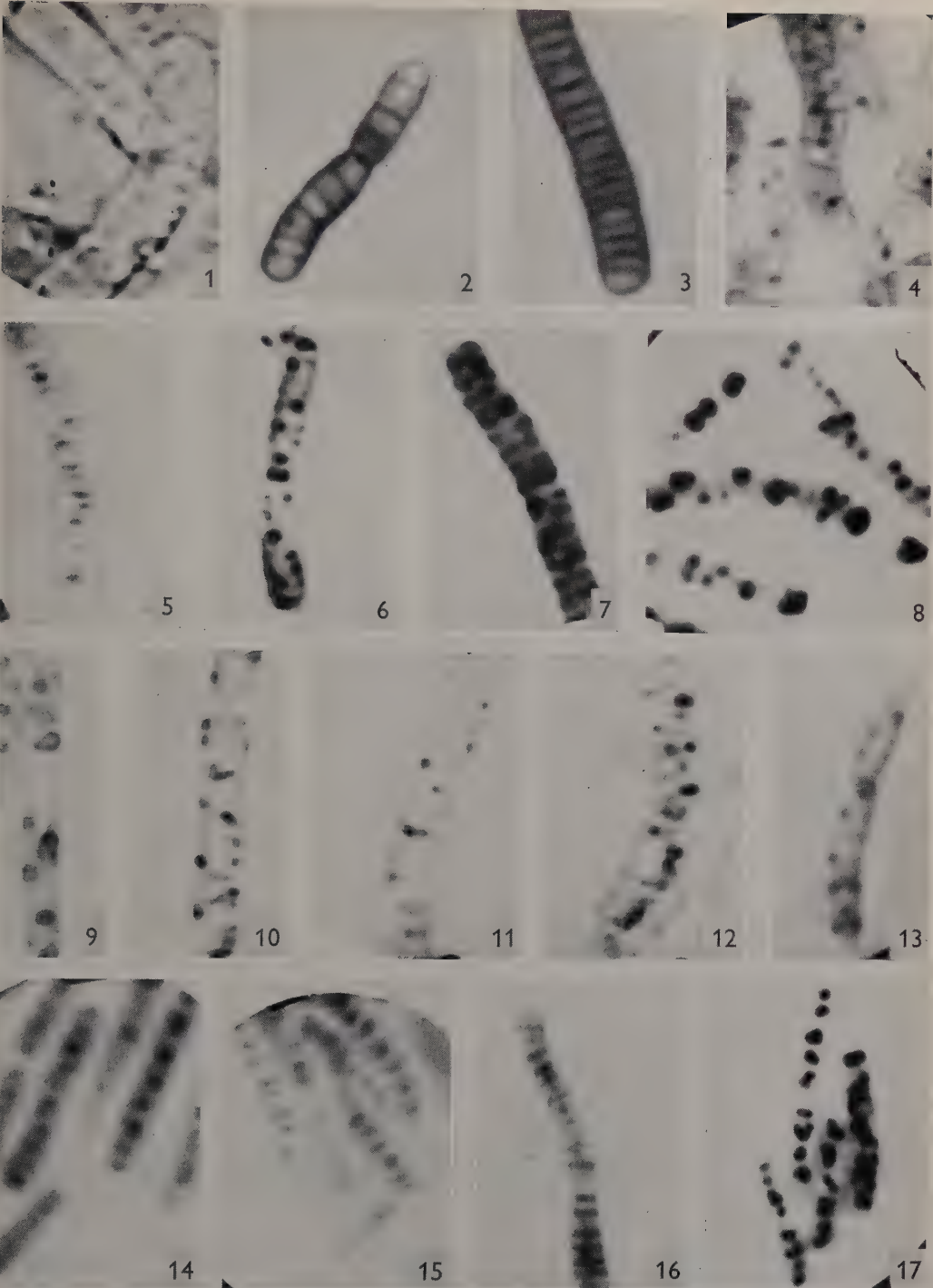
Cytological evidence has been presented indicating the existence of mitochondria in the following genera of bacteria: *Mycobacterium* (Mudd, Winterscheid *et al.* 1951; Winterscheid & Mudd, 1953); *Escherichia* (Mudd, Brodie *et al.* 1951; Hartman, Mudd, Hillier & Beutner, 1953); *Bacillus*, *Micrococcus* (Mudd, Brodie *et al.* 1951); *Salmonella* (Davis *et al.* 1953); *Proteus* (Sorouri & Mudd, 1953); and *Corynebacterium* (Davis & Mudd, 1955); reviewed by Mudd (1953*a*, *b*, *c*; 1954). The cytochemical activities of cytoplasmic granules in *Saccharomyces* were described by Hartman & Liu (1954). The present study demonstrates in *Caryophanon* metabolically active cytoplasmic granules which conform to the definition previously suggested for bacterial mitochondria (Mudd, 1953*b*, *c*). Bisset (1953*b*) has attributed the interpretation of these granules as mitochondria either to the misinterpretation of a variety of artefacts or to failure to recognize the existence of intercellular septa. Bisset's

criticisms have been compared in detail with the cytological evidence and fully answered (Mudd, 1953*a*, *c*; 1954).

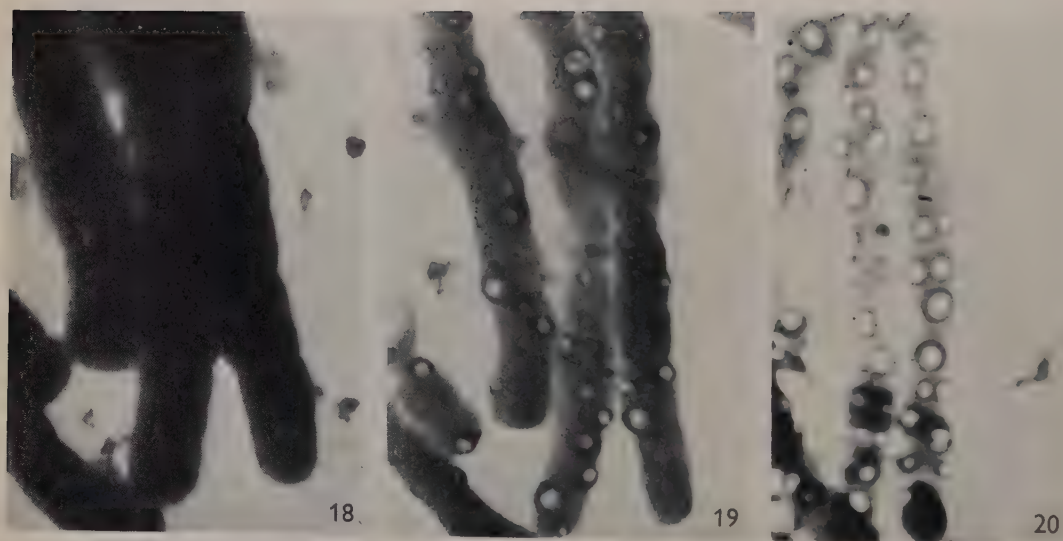
We express our appreciation for helpful suggestions to Drs Philip E. Hartman and John C. Davis.

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T. SALL AND S. MUDD—CYTOLOGY OF *CARYOPHANON LATUM*. PLATE 1



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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. Living cells mounted on wet filter-paper, observed with oblique illumination. Note large eccentric and small peripheral bodies. $\times 4000$.
- Fig. 2. Hale cell-wall stain, wet mount. $\times 4000$.
- Fig. 3. Webb cell-wall stain, wet mount. $\times 4000$.
- Fig. 4. McKinney polysaccharide stain, wet mount. Note the intensely stained structures adjacent to the cross-walls. $\times 3200$.
- Fig. 5. Crystal violet-citric acid (for metachromasy) and Hale cell-wall stain, mounted in Farrant's medium. $\times 4000$.
- Fig. 6. Ebel stain for metaphosphate, wet mount. $\times 4000$.
- Fig. 7. Sudan black B-citric acid stain, mounted in Farrant's medium. $\times 4000$.
- Fig. 8. Pretreated with acetone at room temperature for 1 hr., stained in the Sudan black B-citric acid, mounted in Farrant's medium. $\times 4000$.
- Fig. 9. Menschik (phospholipid), and Hale cell-wall stain, mounted in Farrant's medium. $\times 4000$.
- Fig. 10. Vitally stained preparation with neotetrazolium, wet mount. $\times 4000$.
- Fig. 11. Vitally stained preparation with blue tetrazolium and Hale cell-wall stain, wet mount. $\times 4000$.
- Fig. 12. Vitally stained preparation with triphenyltetrazolium, wet mount. $\times 4000$.
- Fig. 13. Vitally stained preparation with Nadi reagent, wet mount. $\times 3200$.
- Fig. 14. Vitally stained preparation with Janus green B (reduced to the diethyl safranine state). $\times 3200$.
- Fig. 15. Vitally stained preparation with Janus green B (reduced to the leuco safranine state) and Neisser stain. $\times 3200$.
- Fig. 16. Vitally stained preparation with Janus green B (reduced to the diethyl safranine state) and the Hale cell-wall stain, wet mount. $\times 3200$.
- Fig. 17. DeLamater nuclear stain, mounted in Harleco Synthetic Resin. $\times 3200$.

PLATE 2

- Fig. 18. Electron micrograph; impression smear on a collodion membrane, fixed for 1 min. in 2% OsO₄ vapours. Flagella not visible in reproduction. $\times 10,000$.
- Fig. 19. Same cells as shown in fig. 18 after exposure to high intensity electron beam. Note the semi-clear cross-walls and 'blow-outs' which are believed to be metaphosphate deposits. $\times 10,000$.
- Fig. 20. Different field of the same preparation as fig. 19. This field was exposed to a very mild electron beam prior to electron bombardment, in an attempt to avoid 'fixation'. $\times 10,000$.

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A Search for Pathogenic Species of Yeasts in New Zealand Soils

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SUMMARY: One hundred soils from various parts of the South Island of New Zealand were cultivated on an acid medium at 37°. Stockyard soils yielded most yeasts by this treatment, principally strains of *Rhodotorula mucilaginosa* and *Candida parapsilosis*. Few yeasts were isolated from rural soils, and very few from urban soils. *Candida albicans* was recovered from two soils, one from an urban area and one from a stockyard. *Cryptococcus neoformans* was not isolated.

The yeast flora which grew at room temperature (c. 18°) of 8 soils was also examined. One of these, a peat soil, yielded a pure culture of *Candida brumptii*. Species of *Cryptococcus* and *Trichosporon* were dominant in the other seven samples, and their yeast population as a whole was of colourless, capsulated, non-fermenting, starch synthesizing and nitrate utilizing organisms.

Some years ago an experiment was carried out in this laboratory on the ability of the pathogenic yeasts *Cryptococcus neoformans* (Sanfelice) Vuillemin and *Candida albicans* (Robin) Berkhout to survive in soil. Suspensions of cultures which had been recovered from local cases of cryptococcal meningitis and oral thrush were poured over small turf plots which were exposed to the weather. At intervals samples of soil from the plots were cultured on Sabouraud agar of pH 4 and incubated at 37°, conditions which discouraged growth of most members of the normal soil microflora. The pathogens were recoverable for a period of 9 months, at the end of which time it was necessary to destroy the plots. Since then, Emmons (1951) isolated from North American soils four strains of a *Cryptococcus* species pathogenic for mice, which was almost certainly *C. neoformans*, although Lodder & van Rij (1952, p. 375) considered the taxonomic evidence given not sufficiently complete to establish its identity. It did not therefore seem unreasonable to suppose that this pathogen might occur in New Zealand soils, particularly as within the past 5 years four cases of cryptococcal meningitis have been diagnosed here, all in individuals who had spent all their lives within this country.

Candida albicans has been previously reported from a situation unassociated with the mammalian or avian body in one instance, and that not thoroughly authenticated. Negroni & Fischer (1941) isolated *Candida aldoi* (Pereira Filho) Cast. & Jacono from rotting vegetation, and subsequently *C. aldoi* has been placed in synonymy with *C. albicans*. Nevertheless, considering the 50% incidence of *C. albicans* in the human alimentary tract (Marples & di Menna, 1952), and its ability to survive for long periods in the soil, it again seems not unlikely that it should be recoverable from that situation.

The present paper records a search for pathogenic yeasts in 100 soil samples. The culture method described above was used so that only yeasts which would grow at 37° had to be handled. However, a series of eight of the samples were

also cultured at room temperature (c. 18°) and twenty-four yeasts per sample were identified. This was done as a control measure upon the differential culture method used for the entire group. In the sequel the flora which grew at room temperature is referred to as the 18° flora.

MATERIALS AND METHODS

The first eighty soil samples were collected at random from various parts of the South Island of New Zealand. It then appeared that most yeasts were recoverable from stockyard soils, so that the last twenty specimens were gathered from a large stockyard at Invermay Research Station, Mosgiel, Otago. Thirty-eight samples were collected from urban areas, thirty-two from rural areas, and thirty from stockyards.

Four of the eight samples of which both the flora which grew at room temperature and the 37° flora were examined were of tilled soils (*a*, *b*, *c*, *d*) and four of untilled (*e*, *f*, *g*, *h*). Soils (*a*), (*b*), (*c*) and (*f*) were from urban districts, the remainder from the country. The pH values of these samples were as follows: (*a*) pH ± 6.0 , (*b*) pH 6.0–6.5, (*c*) pH 6.5–7.0, (*d*) pH 6.5–7.0, (*e*) pH 5.0–5.5, (*f*) pH 5.0–5.5, (*g*) pH ± 4.5 , (*h*) pH ± 5.0 .

Samples were taken from the top inch of soil, care being taken to avoid including any vegetation. Usually the soil was scooped directly by and into a sterile jar, but in a few cases turves were brought directly into the laboratory for sampling there. In the latter case, the outer layer of soil was scraped away before the final sample was taken in order to avoid excessive contamination. Approximately 1 g. soil with as few rootlets as possible was suspended and well shaken in 10 ml. sterile normal saline and, when the 18° yeast flora was to be investigated, two fivefold dilutions were made from this. Inoculations were made on 100 mm. plates of glucose Sabouraud agar which had been brought to pH 4 immediately before dispensing. The soil suspensions were swilled over the medium, the excess moisture removed with a sterile pipette and the plates incubated upside down. Five plates/soil sample were inoculated with the heaviest suspension and incubated at 37°. In eight soils of which the 18° yeast flora was investigated, a further five plates were inoculated and incubated at room temperature; one of these plates was inoculated with the heaviest suspension and two with each of the dilutions.

Plates held at 37° were incubated for 2–3 days before examination. With the exception of the cultures from one soil sample, all the yeast colonies which appeared were subcultured into glucose yeast extract broth and identified. In this exceptional case there were very many of one type of colony (subsequently found to be of *Saccharomyces cerevisiae* Hansen) and the yeasts from only one of the five plates inoculated were identified. Plates incubated at room temperature were kept for several days, the length of time being dependent upon the amount of mould growth which appeared, and twenty-four yeasts per soil sample were subcultured for identification. In each case they were picked systematically from a single plate or segment of a plate to avoid unconscious selection of large or otherwise conspicuous colonies.

Strains were identified by the keys and criteria of Lodder & van Rij (1952).

Melibiose broth for fermentation tests was prepared by the method of Skinner & Bouthilet (1947).

Particular attention was paid to the 'starch'-producing ability of isolates, particularly those recovered in the investigation of the 18° soil flora. Yeasts were subcultured upon the defined medium of Mager & Aschner (1947), and after 1-2 weeks' incubation at room temperature were flooded with Lugol's iodine; the presence of starch was indicated by blue coloration. Ability to produce starch is the most important character which separates the genus *Cryptococcus* from the genus *Torulopsis*, being invariably present in the former and absent in the latter. Starch production also occurs sporadically throughout certain yeast genera other than *Cryptococcus*. It is present in certain species of *Lipomyces*, *Bullera*, *Candida*, *Trichosporon* and *Rhodotorula* (Aschner & Cury, 1951; Lodder & van Rij, 1952, pp. 31, 334 and 367), and is almost invariably associated with the presence of a capsule or less well organized extracellular slimes, although the reverse is not always true. Wickerham (1952) believes that starch synthesis may be an important key to the phylogenesis of yeasts and other closely related groups, but since this character is present in both the ascosporeogenous *Lipomyces* and the ballistospore-producing *Bullera*, it can surely be of only minor use in tracing relationships.

Problems of identification

The majority of isolates were identified without trouble, but occasional difficulty was experienced. The strains of *Torulopsis aerea* (Saito) Lodder isolated varied from the description given by Lodder & van Rij (1952, p. 413) in that the cells were oval rather than spherical. Connell & Skinner (1953) placed this species in the genus *Cryptococcus* on the grounds that their isolates, and also the authentic strain obtained from Lodder, produced starch when tested by the method of Wickerham (1951). The former authors do not state how they separate their *C. aerius* from *C. albidus* (Saito) Skinner, which it resembles in cell shape, size and biochemical characters. None of the isolates described here as *Torulopsis aerea* synthesized starch.

Two strains isolated at 37° and labelled *Torulopsis famata* (Harrison) Lodder & van Rij were, it was felt, very smooth variants of *Candida parapsilosis* (Ashf.) Langeron & Talice, but as no mycelium could be induced in them, they had to be placed in the former species. Both isolates fermented glucose more strongly than is usual for *Torulopsis famata*, and in addition one produced a bubble of gas in sucrose. The cell shape was oval rather than plump-oval to subglobose, and the cultures on solid media had the shiny yellowish appearance of *Candida parapsilosis*. Neither isolate synthesized starch, and so did not correspond to the *Cryptococcus minor* Poll. & Nann. of Connell & Skinner (1953). Difficulty was experienced in evoking mycelium in a number of other strains of *Candida parapsilosis*.

A new species of *Cryptococcus* (*C. terreus*) was isolated and a description of it has been published (di Menna, 1954a).

RESULTS

Pathogenic yeasts

Three strains of *Candida* (*Monilia*) *albicans* were recovered from two soils. One strain, found in a soil sample from the Dunedin Botanical Gardens, was recognized by its fermentations and pathogenicity. Although it produced mycelium freely, no chlamydospores, which are diagnostic of the species and of the closely related *C. stellatoidea* (Jones & Martin) Lang. & Guerra, were seen upon repeated examination of cornmeal agar cultures. Miss A. v. d. Hoven v. Genderen of the Yeast Division, Centraalbureau voor Schimmelcultures, reported, however, that she was able to find chlamydospores in this strain on cornmeal agar slides after 4 days. Eighty million cells injected intravenously into a rabbit killed the animal in 2 days. The other two strains were recovered from a stockyard soil. They both produced chlamydospores within 24 hr. on cornmeal agar. Doses of 120 million and 80 million cells injected intravenously killed rabbits in 2 days.

Cryptococcus neoformans was not isolated. A strain of *C. laurentii* (Kuff.) Skinner which would grow at 37° was recovered, and a second similar strain was found in an investigation of some soil not connected with this work. Both these yeasts were injected intraperitoneally into mice, but did not establish an infection. Yeasts could not be recovered from the peritoneal cavities of mice killed after the third post-injection day.

Yeasts capable of growth at 37°

A total of 162 strains were isolated from the 100 soil cultures incubated at 37°; of these eleven came from five of the thirty-eight urban soils, thirty-two from nine of the thirty-two rural soils, and 119 from twenty-six of the thirty stockyard soils. As the method used was not a quantitative one, counts are best made, not of the number of strains, but of the number of species isolated per sample.

Rhodotorula mucilaginosa (Jorg.) Harrison was the most common species; it was present in thirty-one soils. *Candida parapsilosis* was the next most frequently found, occurring in thirteen samples. Perfect yeasts were not very common. *Saccharomyces cerevisiae*, *S. delbrueckii* Lindner, and *Pichia fermentans* Lodder were each found in a single sample, and *Debaryomyces subglobosus* (Zach) Lodder & van Rij and *D. kloecckeri* Guill. & Peju were both present in two soils. Details of the numbers and sources of the heat-tolerant yeasts are given in Table 1.

The yeast flora which grew at room temperature (c. 18°)

The 18° yeast flora of seven of the eight soils examined showed a marked consistency of character. The typical yeast of this group was imperfect, colourless and capsulated, did not ferment sugars, utilized nitrate and produced starch on the appropriate medium. Of the 168 yeasts isolated from these seven soils 122 possessed all of the above characteristics, and 146 possessed at least four of them (see Table 2). The dominant yeast was *Cryptococcus*

Table 1. Numbers of soil samples from which heat-tolerant yeasts were isolated

Figures in parentheses are the numbers of cultures isolated.

	Type of soil			
	Urban (38 samples)	Rural (32 samples)	Stockyard (30 samples)	Total (100 samples)
<i>Saccharomyces cerevisiae</i>	.	.	1 (12)	1 (12)
<i>S. delbrueckii</i>	1 (1)	.	.	1 (1)
<i>Pichia fermentans</i>	.	.	1 (1)	1 (1)
<i>Debaryomyces kloeckeri</i>	.	.	2 (6)	2 (6)
<i>D. subglobosus</i>	.	1 (1)	1 (1)	2 (2)
<i>Torulopsis famata</i>	1 (1)	.	2 (2)	3 (3)
<i>T. candida</i> (Saito) Lodder	.	1 (1)	.	1 (1)
<i>Candida albicans</i>	1 (1)	.	1 (2)	2 (3)
<i>C. macedonensis</i> (Cast. & Chalmers) Berkh.	1 (2)	1 (2)	.	2 (4)
<i>C. lipolytica</i> (Harrison) Diddens & Lodder	.	.	1 (1)	1 (1)
<i>C. parapsilosis</i>	2 (4)	1 (6)	10 (18)	13 (28)
<i>Rhodotorula mucilaginosa</i>	1 (2)	6 (19)	24 (72)	31 (93)
<i>R. minuta</i> (Saito) Harrison	.	.	1 (1)	1 (1)
<i>Trichosporon cutaneum</i>	.	.	2 (2)	2 (2)
Failed to grow	.	1 (2)	1 (1)	2 (3)
Soils yielding yeasts	5 (11)	9 (32)	26 (119)	40 (162)

Table 2. Some characteristics of species constituting the yeast flora of the soil which grew at room temperature (c. 18°)

All organisms were colourless except the *Rhodotorula glutinis*.

	Utilizing KNO ₃	Producing 'starch'	Producing capsules	Fermentation of sugars
<i>Debaryomyces hansenii</i>	—	—	—	±
<i>D. kloeckeri</i>	—	—	—	±
<i>D. subglobosus</i>	—	—	—	±
<i>D. nicotianae</i>	—	—	—	±
<i>Cryptococcus laurentii</i>	—	+	+	—
<i>C. albidus</i>	+	+	+	—
<i>C. diffluens</i>	+	+	+	—
<i>C. terreus</i>	+	+	+	—
<i>Torulopsis aerea</i>	—	—	+	—
<i>T. famata</i>	—	—	—	±
<i>Candida tropicalis</i> (Cast.) Berkh.	—	—	—	+
<i>C. humicola</i> (Daszewska) Diddens & Lodler	—	+	+	—
<i>C. brumptii</i>	—	—	—	+
<i>C. scottii</i> Diddens & Lodder	+	—	—	—
<i>Trichosporon pullulans</i>	+	+	+	—
<i>T. cutaneum</i>	—	+	+	—
<i>Rhodotorula glutinis</i>	+	—	+	—

albidus (fifty-eight isolates), and the next most commonly occurring were *C. terreus* (forty-four isolates) and *Trichosporon pullulans* (Lindner) Diddens & Lodder (twenty-six isolates).

The eighth soil (sample *g*), which was from peat at an altitude of 3000 ft. with a pH value of ± 4.5 , produced a pure culture of *Candida brumptii* Lang. & Guerra, a fermenting uncapsulated species which does not assimilate nitrate, or produce a starch-like compound. Pseudomycelium was produced only slowly and in small quantities by all the strains isolated. It is considered likely that, promoted by the acidity of the soil (Langeron & Guerra, 1939), these strains had varied a considerable distance in the S or non-mycelial direction, as had those strains of *C. parapsilosis* and presumptive *C. parapsilosis* mentioned in Methods.

Details of the yeasts isolated are given in Table 3. A greater variety of species was isolated from the tilled soils than from the untilled. The former yielded five to nine species per sample (a total of sixteen species from the group), and the latter one to three species (a total of six).

Table 3. Yeast flora which grew at c. 18° from eight soil samples

	Tilled soils				Untilled soils				Total
	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>e</i>	<i>f</i>	<i>g</i>	<i>h</i>	
<i>Debaryomyces hansenii</i>	.	2	1	3
<i>D. klockeri</i>	.	.	3	3
<i>D. subglobosus</i>	.	2	1	3
<i>D. nicotianae</i>	.	2	1	3
<i>Cryptococcus laurentii</i>	2	.	.	.	1	2	.	.	5
<i>Cr. albidus</i>	8	4	5	.	23	5	.	13	58
<i>Cr. diffluens</i>	.	.	.	1	1
<i>Cr. terreus</i>	4	14	1	1	.	17	.	7	44
<i>Torulopsis aëria</i>	2	2
<i>T. famata</i>	.	.	1	1
<i>Candida tropicalis</i>	.	.	.	3	3
<i>C. humicola</i>	2	4	6
<i>C. brumptii</i>	24	.	24
<i>C. scottii</i>	1	1
<i>Trichosporon pullulans</i>	4	.	8	14	26
<i>Tr. cutaneum</i>	.	.	3	5	8
<i>Rhodotorula glutinis</i>	1	1
Total	24	24	24	24	24	24	24	24	192

DISCUSSION

Pathogenic yeasts

It is believed that this is the first recorded occurrence of *Candida albicans* in the soil, and the second report of its being found unassociated with the mammalian or avian body. It is likely, however, that its apparent rarity away from these sites is not real, but due to lack of work upon the subject, for the preliminary experiments showed that it could at least survive in the soil for a considerable period. Its source in the soil is most likely to be the faeces of various wild and domestic animals in which it is a part of the normal gut flora.

It has been recovered from the alimentary tracts of the European hedgehog and the Australian opossum which are common in both urban and rural areas in New Zealand (di Menna & Parle, 1954), and it has also been isolated from the stomach contents of sheep and pigs (J. N. Parle, unpublished).

It had been hoped that *Cryptococcus neoformans* would be isolated in the course of this survey. Five infections with this organism have been diagnosed in New Zealand between 1948 and 1954. One of these was a lesion of the hand in a South Island man; the other four cases, in all of which the central nervous system was involved, came from a single area, c. 100 miles in diameter, in the North Island. A search is still being made in soils from this area.

The isolation of 37°-tolerant strains of *Cryptococcus laurentii* during this work was of considerable interest in connexion with the pathogenicity of *C. neoformans*. Some earlier work upon the heat tolerance of *Cryptococcus* species had shown that in a group of forty-five strains of various species of this genus only *C. neoformans* could grow at a temperature of over 30° (Table 4). This evidence suggested, as Mager & Aschner (1947) noted, that ability to grow at the temperature of the mammalian body was the factor which conferred pathogenicity upon *C. neoformans*, and was reinforced by the fact that cryptococcosis can only rarely be induced in animals, such as the rabbit, whose body temperatures are at the threshold of the organisms' tolerance (Kuhn, 1939). The inability of strains of *C. laurentii* which grow freely at 37° to produce an infection in mice when administered intraperitoneally suggests that it is not merely heat-tolerance and possession of a capsule which makes *C. neoformans* a pathogen.

Table 4. *The effect of temperature upon growth of some yeast species*

	Strains tested (no.)	Growth at				
		20°	27°	30°	33°	37°
		No. of strains				
<i>Cryptococcus neoformans</i>	3	3	3	3	3	3
<i>C. laurentii</i>	6	6	5	5	0	0
<i>C. albidus</i>	9	9	7	6	0	0
<i>C. diffluens</i>	27	27	25	20	0	0
<i>Debaryomyces hansenii</i>	2	2	nt	nt	nt	0
<i>D. klockeri</i>	18	18	nt	nt	nt	12
<i>D. subglobosus</i>	4	4	nt	nt	nt	4
<i>D. vini</i>	2	2	nt	nt	nt	1
<i>D. nicotianae</i>	3	3	nt	nt	nt	1
<i>D. marama</i>	13	13	nt	nt	nt	0
<i>Torulopsis famata</i>	3	3	nt	nt	nt	2
<i>Trichosporon cutaneum</i>	7	7	7	nt	nt	2

nt = strains not tested at this temperature.

Yeasts capable of growth at 37°

With very minor exceptions, there is no resemblance between this group of yeasts and the yeast flora of the soil which grows at c. 18°. The finding that the numbers of heat-tolerant yeasts isolated was proportional to the amount

of animal pollution suggested that the origin of most members of this group was the alimentary tract of domestic animals. Nevertheless, work in progress by J. N. Parle has so far only partially confirmed this. He has isolated *Pichia fermentans* and *Trichosporon cutaneum* (de Beurm., Gougerot & Vaucher) Ota from the gut contents of sheep and pigs but has found *Rhodotorula mucilaginosa* and *Candida parapsilosis*, which were the most common heat-tolerant species, only very rarely in farm stock. In earlier work (di Menna, 1954*b*), both *C. parapsilosis* and *Rhodotorula mucilaginosa* were isolated from air, the latter in considerable numbers, but although this may explain the occurrence of these species in soil it does not explain why they should be concentrated in soil contaminated by animals.

There is considerable variation in the heat-tolerance shown by different strains of the same species. The effects of a range of temperatures upon the growth of a random selection of species of *Debaryomyces* and *Cryptococcus* (*C. neoformans*, *C. laurentii*, *C. albidus*, *Cryptococcus diffluens* (Zach) Lodder & van Rij, *Debaryomyces hansenii* (Zopf) Lodder & van Rij, *D. kloeckeri*, *D. subglobosus*, *D. vini* Zimmerman, *Debaryomyces nicotianae* Giovannozzi and *Debaryomyces marama* di Menna) and of *Trichosporon cutaneum* are shown in Table 4. This variation explains the apparent anomaly that whilst *Debaryomyces* species and *Trichosporon cutaneum* were isolated from soils cultured only at 37° and from cultures of soils (b), (c) and (d) incubated at room temperature, they were not recovered from cultures of soils (b), (c) and (d) when they were incubated at 37°.

Yeast flora growing at room temperature

The results of this brief survey suggest that the soils of the Otago area, with the exception of peat soils, support a physiologically uniform yeast flora of non-fermenting capsulated colourless species which can synthesize a starch-like compound under certain conditions, and many of which can utilize nitrate nitrogen. The absence of the yeasts of the genus *Lipomyces* from these isolates was curious, for both Den Dooren de Jong in Holland (Lodder & van Rij, 1952, p. 335) and Starkey (1946) in the United States isolated them repeatedly from soils. It is particularly interesting that the described species of *Lipomyces* possess most of the properties which are typical of the soil yeasts isolated here; they are colourless, capsulated, non-fermenting and synthesize starch, although weakly. It is possible that the polysaccharides of the capsules and slimes possessed by soil yeasts may be of some value in improving the physical properties of soil. Swaby (1949) reported that certain gum-producing bacteria were capable of binding together loose soil particles into water-stable aggregates. Yeasts, Swaby stated, did not improve aggregation, but he did not mention which yeasts he used. He found that the majority of bacterial slimes had no aggregating power because they remained water-soluble after drying.

No information appears to be available as to whether or not yeasts possess insoluble polysaccharides. However, it seems reasonable to suppose that a firmly outlined capsule, as is present in yeasts of the genus *Cryptococcus* in both solid and fluid cultures, must contain at least compounds which are

poorly soluble in water. The capsular material found on species of other genera, in particular *Rhodotorula glutinis* (Fres.) Harrison, is less well organized and in Indian ink mounts may be seen streaming away from or even detached from the cell. This material can no longer be regarded as a true capsule, but is rather a cell-secreted mucus, and it seems likely that it may be of a water-soluble type. However, until more information is available it is useless to speculate upon this topic.

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The strain of *Candida albicans* isolated from urban soil and one of the strains isolated from stockyard soil have been deposited with the Centraalbureau voor Schimmelcultures (Yeast Division), Delft, Netherlands.

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The Minimal Nutritional Requirements of Organisms of the Genus *Bordetella* López

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SUMMARY: Eight strains of bronchiseptica, six strains of parapertussis and fifteen strains of pertussis were examined for their minimal nutritional requirements in defined media. All strains showed an absolute requirement for nicotinic acid and no other vitamin was required for growth. Amino acids were essential for parapertussis and pertussis, but bronchiseptica would grow in either a mixture of amino acids, or lactate or citrate. Two old laboratory strains were exceptional in that they could utilize either glutamic acid, α -ketoglutaric acid, citrate, lactate, succinate or pyruvate. The amino acid requirements of the three species were relatively simple and showed some similarities. Bronchiseptica would grow in a mixture of glutamic acid, proline and leucine, while parapertussis required added cystine and methionine, and pertussis required in addition alanine, asparagine and serine. In simple amino acid mixtures glutamic acid was essential, but was replaceable by α -ketoglutaric acid. Nutritionally the species are very similar, but are quite different from the *Haemophilus influenzae-parainfluenzae-canis*, or the *Brucella abortus-suis-melitensis* groups of organisms. The nutritional evidence supports the already impressive evidence on other grounds that these three groups should be classified separately. If generic status is given to one group it should be given to all three. López (1952) has proposed a new genus *Bordetella*, consisting of the species *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, and this seems a reasonable solution to the present anomalous position. The three species can be differentiated by tests for inhibition. Thus *B. bronchiseptica* is the only species not inhibited by 2.0 % citrate and *B. pertussis* the only species inhibited by colloidal copper sulphide.

The Parvobacteriaceae have been subdivided into a multiplicity of genera, primarily on grounds of animal pathogenicity. Many of the genera consist of only a few pathogenic species; in some instances all the species in a genus produce similar diseases in the field or in experimental animals. Since pathogens are highly specialized species it has usually been possible to devise a number of positive or negative diagnostic tests for their identity, tests which are not so readily available for the whole range of bacteria. To some extent these tests have obscured the underlying basis of pathogenicity used for their classification. This classification is, of course, a reflexion of the historical development of bacteriology and has been evolved to satisfy the practical needs of human and veterinary medicine. Even if comparative studies made it possible to present a more aesthetically satisfying classification on general biological lines it would almost certainly be unacceptable at the present time to those workers most interested in this group of organisms. However, the existing system of classification introduces two difficulties that are pertinent here. First, it is difficult to introduce a species into such an arbitrary classification. Thus, when it became apparent that bronchiseptica should be in this group, it had to be classified as either *Brucella bronchiseptica* or *Haemophilus*

bronchisepticus, whereas it was clear that the generic definitions really excluded this species from either genus. The second difficulty is that as further information becomes available the generic definition may exclude established species. Thus Fildes (1923) showed that the generic definition excluded the pertussis organism from the genus *Haemophilus* where it had become well established. In practice any widening of the generic definition introduces chaos into the whole group. For a long time it has been realized that bronchiseptica, parapertussis and pertussis have many common distinctive characters and cannot reasonably be classified in any of the existing genera. López (1952) summarized much of this evidence and proposed a new genus *Bordetella* consisting of these three species. The nutritional studies reported here support this view and this classification has been adopted.

METHODS

Strains. All strains were from the laboratory collection where they had been maintained as freeze-dried cultures. They were all examined for purity and identity by the relevant bacteriological and serological tests.

Nutritional requirements. The methods used were similar to those described by Knight & Proom (1950); for a detailed description reference should be made to that publication, but for convenience the salient points are here given. The usual precautions were taken to exclude contamination by unwanted nutrients. The starting-point for an experiment was a freeze-dried culture from the laboratory collection. At least five subcultures were made on defined media to exclude the possibility of carry-over of essential nutrients. At the conclusion of each experiment the final culture was checked for purity and identity. To exclude the possibility of selection of less exacting nutrients the original strain from the collection was tested for its ability to grow on the minimal medium finally found to be adequate.

Media were dispensed in 5 ml. lots in 6 × 1 in. rimless boiling tubes closed by loosely fitting aluminium caps. Cultures were incubated at 37° in a sloped position to give good aeration. The initial inoculum was taken from an agar slope with a small loop and subsequent inoculations were made with a small loop.

Media

Medium 6 (ammonia basal medium). KH_2PO_4 , 1.5 g.; $(\text{NH}_4)_2\text{HPO}_4$, 7.0 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.3 g.; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.04 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.025 g.; ammonium molybdate 0.002 g.; dissolved in distilled water to 1 l.; pH adjusted to 7.6 with NaOH. Solution boiled, filtered through paper, and sterilized for 20 min. at 115°.

Medium 7 AA (seven amino acid medium). Medium 6 supplemented with the following amino acids in the concentrations shown (in mg./ml.). L-asparagine, 0.4; L-proline, 0.1; L-leucine, 0.57; DL-alanine, 0.38; L-glutamic acid, 1.4; DL-serine, 0.12; DL-methionine, 0.06. The amino acid mixture was prepared separately by solution in distilled water at 10 times these concentrations. It was gassed with H_2S , filtered through paper and sterilized at 115° for 20 min.

It was then added aseptically to medium 6 to give the required concentration of amino acids.

Medium SC 2 C (complex amino acid medium). Medium 6 supplemented with the following amino acids in the concentrations shown (in mg./ml.). DL-alanine, 0.38; DL-aspartic acid, 0.89; L-arginine HCl, 0.3; L-cystine, 0.02; L-glutamic acid, 1.4; glycine, 0.17; L-histidine HCl, 0.24; DL-isoleucine, 0.76; L-leucine, 0.57; L-lysine HCl, 0.24; DL-methionine, 0.06; L-proline, 0.11; DL-serine, 0.12; DL-threonine, 0.10; L-tyrosine, 0.06; DL-valine, 0.15. The amino acid mixture was prepared separately by solution in distilled water at 10 times the required concentration. It was gassed with H_2S , filtered through paper, sterilized at 115° for 20 min., and added aseptically to medium 6 to give the required concentration.

Medium SC2H. Identical with medium SC2C except that the amino acid mixture was not gassed with H_2S and was sterilized by Seitz filtration.

Medium SC2F. Identical with medium SC2C except that all the amino acids were in the L configuration. Also L-phenylalanine, L-tryptophan were included to give final concentrations in the medium of 0.1 and 0.03 mg./ml. respectively; gassed with H_2S .

Medium SC2G. Identical with medium SC2F except that the amino acid mixture was not gassed with H_2S and was sterilized by Seitz filtration.

Medium 11 (hydrolysed casein medium). Medium 6 supplemented with 'vitamin-free' hydrolysed casein (Allen and Hanburys Ltd.) to give an additional 0.1% total nitrogen. The hydrolysed casein solution was prepared at 10 times the required concentration, and the pH value adjusted to 7.5 with NaOH. The solution was boiled and filtered through paper, sterilized at 115° for 20 min. and added aseptically to medium 6.

Hornibrook's amino acid mixture. Medium 6 supplemented with the amino acid mixture used by Hornibrook (1940). The amino acid mixture was used in the concentrations shown (in mg./ml.). DL-glutamic acid, 1.8; L-tyrosine, 0.2; glycine, 0.045; L-proline, 0.4; L-histidine, 0.1; DL-arginine, 0.2; L-cystine, 0.01. The amino acid mixture was prepared in distilled water at 10 times the final concentration, sterilized by Seitz filtration and added aseptically to medium 6.

Simple amino acid mixtures. When single amino acids or mixtures of two or three were used, they were added to medium 6 to give the same concentrations as in medium SC2C. The amino acids were dissolved in distilled water at 10 times the final concentration, sterilized for 20 min. at 115° and added aseptically to medium 6.

Medium 14 (citrate+acetate medium). From the medium described by Henderson & Snell (1948): sodium citrate, 2.0%; sodium acetate, 1.0%; NH_4Cl , 0.3%; K_2HPO_4 , 0.5%; 2.0% (v/v) trace metal solution C from Henderson & Snell (1948).

Soluble starch. A stock 1.5% solution of Analar grade soluble starch (British Drug Houses Ltd.) dissolved in boiling water and sterilized 20 min. at 115°. The solution was added aseptically to the otherwise complete medium to give a final concentration of 0.15%. One bottle of a batch was reserved for this

purpose. When tested with *Lactobacillus casei* no appreciable quantities of any of the growth factors required by this species were found in the sample. It was therefore assumed that the addition of starch did not result in the addition of growth factors.

RESULTS

The minimal nutritional requirements of the three species are summarized in Table 1. The detailed results with each species are given below.

Table 1. *The nutritional requirements of Bordetella spp.*

Medium	<i>B. bronchiseptica</i>	<i>B. parapertussis</i>	<i>B. pertussis</i>
1. Ammonia basal salt medium + complex amino acid mixture (SC2H) + nicotinic acid	+	+	+
			(phase I strains required added starch)
2. Medium 1 without nicotinic acid	—	—	—
3. Ammonia basal salt medium + seven amino acid mixture (7AA) + nicotinic acid	+	+	—
			(+ with added cystine)
4. No. 3 without glutamic acid	—*	—	—
			(— with added cystine)
5. No. 4 with α -ketoglutaric acid	+	+	+
			(with added cystine)
6. Ammonia basal salt medium + nicotinic acid + glutamic acid + proline + leucine	+	—	—
		(+ with added cystine, methionine)	
7. Acetate citrate (medium 14) + nicotinic acid	+†	—	—
8. Ammonia basal salt medium + nicotinic acid + either α -ketoglutarate, acetate, lactate, citrate, glucose, glutamic acid or pyruvate	(a) Old laboratory strains + with all except glucose. (b) Freshly isolated animal strains + with lactate or citrate only	—	—

* One animal strain grew.

† One animal strain would not grow.

Bordetella bronchiseptica

Eight strains of *B. bronchiseptica* were studied: four strains isolated from the lungs of dogs with secondary infection following dog distemper, one strain from the lung of a ferret and one from guinea-pig lung. These strains had been freeze-dried shortly after isolation. The remaining two were very old laboratory strains of unknown origin which had been subcultivated on laboratory media for many years before freeze-drying.

The eight strains grew on the complex amino acid medium (SC2C) and in the seven amino acid medium (7AA) with nicotinic acid (0.5 μ g./ml.) as an essential nutrient. The requirement for nicotinic acid was absolute, and this growth factor had to be added to all media to permit growth.

The growth requirements of the two old laboratory strains were simpler. They would grow in the ammonia basal medium (6) plus nicotinic acid, with the addition of either glutamic acid, lactate, pyruvate, citrate or α -ketoglutarate. Glucose would not support growth; this was to be expected as the species does not ferment glucose. In simple mixtures of amino acids glutamic acid was essential; for example, the strains would grow in a mixture of glutamic acid, proline and leucine but would not grow with proline and leucine alone.

The growth requirements of the remaining six strains were more complex and rather variable. With the complex amino acid medium (SC2C) minus glutamic acid, five strains grew poorly and occasionally failed to grow on serial subculture. In most experiments after growing poorly for a few subcultures the strains improved in growth which eventually became as good, or nearly as good, as in the complete medium. One strain from the start grew equally well in both media. With the seven amino acid medium (7 AA) minus glutamic acid two strains failed to grow, while two strains grew poorly for a few subcultures and then died out. One strain grew poorly but growth eventually improved and one strain grew equally well on both media. Three strains grew well in a mixture of glutamic acid, proline and leucine, the other three strains grew poorly and occasionally faded out on serial subcultivation. None of the six strains would grow on proline + leucine, and all six strains gave maximal growth with a mixture of glutamic acid, proline, leucine, cystine and methionine.

Five of the six strains grew in the citrate + acetate medium (14). Growth was at first poor, but after a few subcultures improved and became as good, or nearly as good, as in the amino acid medium. The remaining strain grew in medium 14 after the addition of the seven amino acid mixture. Acetate, α -ketoglutarate, lactate, citrate, pyruvate and succinate in 1.0% concentrations were tested as single carbon and energy sources. The strain that would not grow in medium 14 would not grow in any of the above media. The results with the remaining strains were variable, but followed a definite pattern. When growth occurred it was poor and remained so for one or two subcultures and then either the culture died or growth improved until after five or six subcultures it became as good, or nearly as good, as with the amino acid media. As carbon sources only lactate or citrate supported growth; α -ketoglutarate, acetate, pyruvate and succinate were ineffective. However, since the experiments with lactate and citrate showed clear evidence of selection it is possible that variants could be selected that would grow on all the above substrates. The experiments, however, clearly showed that lactate and citrate were more readily utilized.

α -Ketoglutarate could replace glutamic acid in any of the amino acid media. For example, strains that grew poorly on a mixture of glutamic acid, proline and leucine, grew poorly in a mixture of α -ketoglutarate, proline and leucine, whereas those strains that grew well in the first medium grew well in the second.

Bordetella parapertussis

Six strains of *B. parapertussis* were studied, all having been isolated from cases of whooping cough. Four of the six strains had been freeze-dried within a few subcultures of isolation; the remaining two strains had been subcultured on Bordet-Gengou medium for some years before drying.

The six strains grew in the complex amino acid medium (SC2C) or in the seven amino acid medium (7 AA) with nicotinic acid (0.5 $\mu\text{g./ml.}$) as an essential nutrient. The requirement for nicotinic acid was absolute, and this growth factor had to be added to all media to permit growth. Glutamic acid appeared to be essential, since the six strains would not grow in medium SC2C minus glutamic acid or in medium 7 AA minus glutamic acid. The strains would grow poorly for a few subcultures in glutamic acid + proline + leucine, but they could not be maintained in serial subculture therein. They could be subcultivated indefinitely with added cystine and methionine. Glutamic acid alone would not support growth.

Amino acids were essential for growth and none of the strains would grow on the ammonia + citrate + acetate medium (14). Moreover, the concentration of citrate (2.0 %) in medium 14 inhibited the growth of all strains when added to amino acid media. Acetate, α -ketoglutarate, citrate, lactate, pyruvate and succinate were tested as single sources of carbon and energy but were unable to support growth, although in the 1.0 % concentrations used they were not inhibitory in the seven amino acid medium (7 AA). Citrate and α -ketoglutarate but not lactate, succinate or pyruvate, could replace glutamic acid in the amino acid media.

Bordetella pertussis

Fifteen strains of *B. pertussis* were studied. Thirteen were virulent phase I strains (Leslie & Gardner, 1931) isolated from cases of whooping cough and freeze-dried within a few subcultures of isolation. The remaining two strains were selected avirulent phase IV variants able to grow on nutrient agar.

All thirteen virulent strains would grow and could be maintained in serial subcultivation in some batches of 'vitamin-free' hydrolysed casein (11) with added nicotinic acid (0.5 $\mu\text{g./ml.}$) and with 0.15 % soluble starch to adsorb inhibitory fatty acids present in the medium and produced during growth (Pollock, 1947). The requirement for nicotinic acid was absolute and this growth factor had to be added to all media to allow growth. The two avirulent strains had similar requirements except they would grow in the absence of starch.

With the first inoculum from Bordet-Gengou medium the strains sometimes grew on the above medium when the hydrolysed casein was replaced by the complex amino acid medium (SC2C), but in all cases the cultures died after one or two subcultures. The failure to grow in the absence of 'vitamin-free' casein hydrolysate might have been due to a missing growth factor, to an inhibitor present in medium SC2C or to the balance of amino acid concentrations.

With the first batch of hydrolysed casein used there was no clear evidence that medium SC2C was inhibitory in the presence of hydrolysed casein.

Moreover, medium SC2C was not inhibitory to the related species *Bordetella parapertussis* or *B. bronchiseptica*. A search was therefore made for a missing growth factor. A very wide range of substances was tried, including a preparation of streptogenin made from crystalline insulin by tryptic digestion according to the method described by Sprince & Wooley (1945). None of these substances, individually or in combination, when added to medium SC2C would permit growth. Attempts to isolate a growth-promoting fraction from hydrolysed casein by paper chromatography or by fractionation in an all-glass Craig separator were uniformly unsuccessful.

The unlikely event that failure to grow was due to the relative proportions of amino acids present in the two media was investigated. A batch of medium SC2C was prepared, and by arbitrary addition of various amino acids was matched visually by two-dimensional paper chromatography until it was identical with hydrolysed casein. No growth was obtained with this medium.

Medium SC2G was prepared in which all the amino acids were present in the L-form. Since this medium did not support growth the failure to grow in medium SC2C was therefore not due to an inhibition by D-amino acids.

A number of different batches of hydrolysed casein were used and batch variation was found to be considerable. Some batches supported growth, others failed to support growth or growth died out on the first subculture. Strain variation was also evident. Some batches supported growth of phase IV but not phase I strains, while others supported the growth of phase IV and a limited number of phase I strains. With some batches of hydrolysed casein growth was obtained at the usual concentration equivalent to a total nitrogen (TN) of 0.1 g./l., but was poor at a TN of 0.2 g./l., and there was no growth at 0.4 g./l.; this suggested the presence of an inhibitor and this possibility was reconsidered.

Two recent publications (Schuhardt, Rode, Oglesby & Lankford (1952) and Woiwod (1954)), appeared relevant and suggested that the inhibitor might be colloidal sulphur or colloidal copper sulphide. It was found that media prepared from unsatisfactory batches of hydrolysed casein permitted growth of all strains of *Bordetella pertussis* when sterilized by Seitz filtration instead of by heat. It was also found that the addition of cystine, sterilized by filtration,

Table 2. *The inhibitory effect of citrate and colloidal copper sulphide*

Medium	Strain		
	<i>bronchiseptica</i>	<i>parapertussis</i>	<i>pertussis</i>
Complex amino acid medium (SC2H)*	+	+	+
SC2H+2.0% citrate	+	—	—
SC2H+colloidal copper sulphide	+	+	—
Complex amino acid medium (SC2C)†	+	+	—
SC2C filtered through membrane	.	.	+
SC2C+appropriate dilution of β -mercaptopropionic acid	.	.	+

* SC2H sterilized by Seitz filtration and not gassed with H_2S .

† SC2C prepared by gassing with H_2S and sterilized by heat. — = no growth; + = growth; . = not tested.

completely annulled the inhibition by the heat-sterilized medium. These results were consistent with the inhibitor being colloidal sulphur, as Woiwod (1954) showed that inhibition by colloidal copper sulphide was not annulled by cystine.

Chemically defined media SC2H and SC2G, identical with SC2C and SC2F except that they were not gassed with H_2S and were sterilized by Seitz filtration instead of by heat, supported the growth of all strains of *Bordetella pertussis* tested. It was thus clear that failure to grow in the complex amino acid medium SC2C was due to an inhibitor which had been formed during the preparation of the medium. The inhibitory medium SC2C was filtered through membrane filters (Apis Engineering and Research Ltd.) of pore size 200–500 m μ . Two phase IV strains and three of five phase I strains were maintained in serial subculture in the ultrafiltered medium. This was regarded as conclusive evidence that the inhibitor was particulate. Filters of smaller pore size were not used because of the difficulty of filtering sufficient medium for test. Attempts were made to annul the inhibitory effect in medium SC2C with a solution of Seitz-filtered cystine. The results were not so clear-cut as with hydrolysed casein. Phase IV strains and many phase I strains would grow in serial subculture, but a number of phase I strains failed to grow.

Attempts were made to see whether those strains which failed to grow with added cystine would grow after the addition of substances shown by Woiwod (1954) to annul the inhibitory action of colloidal copper sulphide. The results given in Table 3 show that an appropriate concentration of β -mercapto-

Table 3. *Reversal of inhibition present in medium SC2C by β -mercaptopropionic acid (β -m.p.a.)*

Medium	% β -m.p.a. in medium	Growth of <i>B. pertussis</i> after		
		48 hr.	72 hr.	96 hr.
Complex amino acid medium SC2H (non-inhibitory medium)	0.25	—	—	—
	0.125	—	—	—
	0.06	—	—	—
	0.025	—	tr.	±
	0.01	tr.	±	+
	None	+	+	++
Complex amino acid medium SC2C (inhibitory medium)	0.25	—	—	—
	0.125	—	—	—
	0.06	—	—	—
	0.025	tr.	+	++
	0.01	+	++	++
	None	—	—	—

— = no growth; tr., ±, +, ++ = amount of growth.

propionic acid promoted growth. The strain could not be maintained in serial subculture because the toxicity of media containing β -mercaptopropionic acid increased on standing. Bisulphite or β -mercaptopropanol either did not annul the inhibitor or the concentration necessary for annulment was itself toxic. β -Mercaptopropanol was very toxic for *Bordetella pertussis* and inhibited growth in medium SC2H at a concentration of less than 0.01%. It is possible

that these results could be explained on the basis of variation in sensitivity of different strains of *B. pertussis* to colloidal sulphur. It seems more likely, however, that both colloidal sulphur and colloidal copper sulphide were present but only sufficient colloidal copper sulphide to inhibit the more sensitive strains.

Colloidal copper sulphide (10 mg. Cu/l.), provided by Dr A. J. Woiwod, inhibited the growth of *Bordetella pertussis* in medium SC2H at a dilution of 1/64. At a dilution of 1/4 it did not inhibit *B. parapertussis* or *B. bronchiseptica* in any of the media tested including a simple mixture of amino acids. A limited number of experiments showed clearly that the inhibitory effect of colloidal copper sulphide varied both with the strains and the medium to which it was added. Phase IV strains were more resistant than phase I strains, and there was considerable variation in the susceptibility of different phase I strains. The more complex the medium the less was the inhibitory effect of added sol.

The amino acid requirements of *Bordetella pertussis* were examined in some detail, and to avoid inhibitory effects all the amino acids were sterilized by Seitz filtration. All strains grew in serial subculture in the complex amino acid medium (SC2H) or in the seven amino acid medium (7AA) with added cystine. Since both these media contained no utilizable carbohydrate the organism obtained its energy by metabolism of amino acids. Strains of *B. pertussis* grew reasonably well in medium SC2H minus glutamic acid and in this medium glutamic acid was not essential as was the case with *B. parapertussis*. However, glutamic acid was essential with the simpler amino acid medium 7AA plus cystine. With phase I strains the glutamic acid could be replaced by α -ketoglutarate or succinate but not by lactate or pyruvate. Citrate could replace glutamic acid with a minority of phase I strains. With the two phase IV strains glutamic acid could be replaced by citrate, α -ketoglutarate, or succinate but not by lactate or pyruvate. These results are different from those obtained with *B. parapertussis*.

Bordetella pertussis sometimes grew for one or two subcultures in very simple amino acid mixtures, but for serial subcultivation the requirements were more complex. Strains could grow up to five subcultures in Hornibrook's (1940) amino acid mixture but required added alanine for indefinite subcultivation. It was not practicable to determine all the combinations of amino acids that would support growth, but it seemed likely that medium 7AA + cystine or Hornibrook's (1940) amino acid mixture + alanine represented two of the simplest combinations capable of supporting growth. Experiments were performed in which single amino acids were omitted from the above mixtures and from medium SC2H. It was not always easy to interpret the results, and the experiments were very laborious since up to ten serial subcultures had to be made with each combination of amino acids. Cystine appeared essential for growth and could not be replaced by methionine or any other mixture of amino acids. Proline, leucine or alanine, if not essential, could only be replaced by complicated mixtures of amino acids. With simple amino acid mixtures glutamic acid was essential but could be replaced by an alternative source of energy such as α -ketoglutarate. However, a mixture of the above five amino

acids (cystine, proline, leucine, alanine and glutamic acid) was not sufficient to maintain all strains in serial subculture and a mixture of three or more other amino acids had to be added. It seemed that almost any combination of three or four different amino acids would suffice, and the analysis was not further continued.

DISCUSSION

Hornibrook (1939) described a semi-defined medium consisting of inorganic salts, hydrolysed casein, cystine and soluble starch that supported the growth of freshly isolated strains of *Bordetella pertussis*. Yeast extract, though not essential, improved growth. Shortly afterwards Hornibrook (1940) showed that when the inoculum from a culture on Bordet-Gengou medium was small yeast extract became essential, and that *B. pertussis* would grow in a medium consisting of soluble starch, salts, a mixture of seven amino acids, and nicotinic acid. This paper clearly demonstrated the requirement for nicotinic acid but gave no information on the course of events on serial subculture. Later workers have confined their attention to modifications of Hornibrook's medium to give improved growth for the production of pertussis vaccines for active immunization. Such modifications have been in the composition of the salt mixture and in the addition of accessory nutrients such as yeast dialysate or liver extract. With these media it has been common experience that variations in the method of casein digestion, and even batch variations when using the same process, influences considerably the amount of growth obtained. It is possible that these variations might, in part, be due to variations in the amount of inhibitory material present. No reports have been observed in the literature dealing with the exact nutritional requirements of *B. parapertussis*.

While this work was in progress Ulrich & Needham (1953) reported on the nutritional requirements of *Bordetella bronchiseptica* in a paper dealing with the differentiation of this species from *Alcaligenes faecalis*. Sixteen of eighteen strains required nicotinic acid, four strains required in addition pantothenate and two strains did not require any vitamin. In the present study nicotinic acid was required by all the strains of *Bordetella bronchiseptica*, *B. parapertussis* and *B. pertussis*, and was the only vitamin necessary for growth. It is of interest to note that the two very old strains of *B. bronchiseptica* which had been subcultured on artificial media for at least 20 years, as well as the two strains of *B. parapertussis* subcultivated on Bordet-Gengou medium for several years and the two selected variants of *B. pertussis* which grew on nutrient agar, still required nicotinic acid. Knight & Proom (1950) have already commented on the similarity of vitamin requirements of old laboratory strains and fresh isolates in the genus *Bacillus*. Proom & Woiwod (1951) examined a large number of *Proteus* strains, some of which were very old laboratory cultures, and all showed the same basic vitamin requirements. There is cumulative evidence suggesting that the basal vitamin requirements of a bacterial species are among one of its more constant characters. Although variants with more complex vitamin requirements can be produced in the laboratory or may occur in nature, strains less exacting than those of the basal species pattern would seem to be rare.

Bordetella pertussis, *B. paraptussis* and *B. bronchiseptica* do not metabolize glucose and obtain their energy and carbon from either organic acids or amino acids. They do not exhibit the same absolute requirement for specific compounds as is the case with the vitamin requirement for nicotinic acid. Rather they form a graduated series of strains and species having increasing nutritional complexity.

The two old laboratory strains of *Bordetella bronchiseptica* can utilize either glutamic acid, lactate, citrate, pyruvate or α -ketoglutarate. These strains had the most active metabolism of those tested. Chromatograms of cultures grown in hydrolysed casein showed that all the amino acids had been metabolized except for some occupying the position of aspartic acid in the single dimensional chromatogram. They show the typical pattern described by Proom & Woiwod (1949) for biochemically active Gram-negative organisms. With other strains of *B. bronchiseptica* and strains of *B. paraptussis* and *B. pertussis*, the chromatograms showed that cystine, serine, glutamic acid, alanine, proline and leucine had been metabolized. These chromatographic results were a rough guide in the selection of simple amino acid mixtures likely to support growth.

Apart from the two old laboratory strains, five recently isolated strains of *Bordetella bronchiseptica* grew in the absence of amino acids. It was evident that selection occurred before the strains grew readily. There seems no obvious reason why citrate or lactate should be more readily utilized than pyruvate or α -ketoglutarate, particularly since in the amino acid mixture glutamic acid could be replaced by α -ketoglutarate. The remaining strain of *B. bronchiseptica* would not grow in the absence of amino acids. With this strain the high concentration of citrate (2%) in medium 14 was not inhibitory when added to the seven amino acid medium.

The amino acid requirements of the three species show some similarities. *Bordetella bronchiseptica*, in the absence of organic acids, requires glutamic acid, proline and leucine; cystine and methionine are also required to give good growth with all strains. *B. paraptussis* grows poorly with glutamic acid, proline and leucine and for serial subculture requires added cystine and methionine. *B. pertussis* will grow for a few subcultures in Hornibrook's (1940) amino acid mixture but for serial subculture requires added alanine. Alternatively, this species can be maintained in serial subculture in the seven amino acid mixture (7AA) with added cystine or in the complex amino acid medium (SC2H).

Since the three species do not utilize glucose or, with the exception of *Bordetella bronchiseptica*, organic acids, it is not surprising that glutamic acid is specially important, since it is the acid most likely to be used as a source of energy. The chromatographic evidence showed that it was the amino acid most rapidly and completely metabolized in growing cultures. In simple mixtures of amino acids it was found to be essential, but with more complex mixtures the experimental evidence was not so conclusive. Certainly it seems to be preferred, but it was possible here that other amino acids, either alone or in combination, could replace it as an energy source. However, purity is a

relative term and since the actual amounts of amino acid necessary for growth are very small it is unwise to assume that the complex mixtures of amino acids were free from traces of contaminating glutamic acid.

It is usually assumed that when glutamic acid is used as a source of energy it is first converted by the organism to α -ketoglutaric acid. In fact, with all strains of *Bordetella bronchiseptica*, *B. parapertussis* and *B. pertussis*, α -ketoglutarate could replace glutamic acid in simple mixtures of amino acids. However, many strains of *B. bronchiseptica* utilized α -ketoglutarate only in the presence of amino acids but would utilize citrate or lactate in the absence of amino acids. The samples of citrate and lactate used were carefully examined and found free from traces of amino acids.

The investigation of the nutrition of *Bordetella pertussis* was hampered by inhibitory materials formed in the preparation of the defined media. The experiments suggested that the inhibitor present in the heat-sterilized hydrolysed casein was colloidal sulphur. The inhibitor present in the defined amino acid medium was either colloidal sulphur or a mixture of colloidal sulphur and colloidal copper sulphide. The procedures responsible for their production were gassing with H_2S to remove heavy metals and sterilization by heat. The inhibitors were only produced in the presence of cystine, and their production could be eliminated either by sterilization by filtration without gassing with H_2S , or by the addition of cystine as a Seitz-filtered solution to the otherwise complete medium. The observation that inhibitory effects may arise during the preparation of defined media is of course very old, and these particular inhibitors have recently been examined in some detail by Schuhardt *et al.* (1952) and by Woiwod (1954). These results are reported as an example of some of the difficulties that may arise in the preparation of defined media for nutritional studies. It is not always easy to decide whether failure to grow is due to the presence of an inhibitor or the absence of a growth factor. Moreover, the inhibitory effects became progressively greater as the medium became simpler in composition.

It is interesting to note that the three species could be differentiated on the basis of tests for growth inhibition. *Bordetella bronchiseptica* is not inhibited by 2.0% citrate in the presence of amino acids, whereas *B. parapertussis* and *B. pertussis* are inhibited. *B. pertussis* is the only species inhibited by colloidal copper sulphide.

These results show very clearly that nutritionally *Bordetella pertussis*, *B. parapertussis* and *B. bronchiseptica* are similar. All show an absolute requirement for nicotinic acid and require no other vitamin for growth. In simple amino acid mixtures all three species use glutamic acid as a source of energy and this acid can be replaced by α -ketoglutaric acid. The amino acid mixtures necessary for growth are similar, forming a series of slightly increasing complexity. The nutritional requirements of these species are quite different from those of *Haemophilus influenzae-parainfluenzae-canis* group in that they do not require haemin or phosphopyridine nucleotide (X and V factors) and from the *Brucella abortus-suis-melitensis* group in that they do not require thiamine, biotin or pantothenate. These results support the already

impressive evidence on physiological, serological and toxigenic grounds that if generic status is given to one of these groups it should be given to all three. López (1952) has proposed a new genus, *Bordetella*, to include the species *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, and in a footnote stated that this classification will be adopted in the seventh edition of *Bergey's Manual of Determinative Bacteriology*. This seems a reasonable solution to the present anomalous taxonomic position.

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Bactericidal Activity of Streptomycin and Isoniazid in Combination with *p*-Aminosalicylic Acid against *Mycobacterium tuberculosis*

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SUMMARY: The bactericidal activity of streptomycin, isoniazid and combinations of streptomycin and isoniazid against tubercle bacilli growing in Tween albumin medium was measured with and without the addition of *p*-aminosalicylic acid (PAS). When the concentrations of these compounds were about 4 to 16 times their minimal inhibitory concentrations, PAS did not influence this activity, but it was slightly increased when the concentrations were 10 times higher (equal to peak serum concentrations in treated patients). Combinations of the low concentrations of PAS + streptomycin or PAS + isoniazid usually only delayed the emergence of drug-resistant bacilli, whereas combinations of the higher concentrations suppressed their growth.

Lehmann (1946) reported that *p*-aminosalicylic acid (PAS) inhibited the growth of *Mycobacterium tuberculosis*. This inhibition was, however, only partial and PAS was less effective than streptomycin or isoniazid in the treatment of both human pulmonary tuberculosis (Medical Research Council, 1950) and experimental tuberculosis in mice and guinea pigs (McClosky, Smith & Frias, 1948; Swedburg, 1949; Swedburg & Widstrom, 1948; Steenken & Wolinsky, 1950). PAS is now usually used in treatment together with streptomycin or isoniazid. The main purpose of this combined therapy is to prevent the emergence of drug-resistant strains of tubercle bacilli which have occurred in about two-thirds of cultures from patients with acute pulmonary tuberculosis who have been treated with streptomycin or isoniazid alone and in about a quarter of cultures from those treated with PAS alone over a period of 3 months (Medical Research Council, 1948, 1950, 1953*b*). Combined treatment with either streptomycin and PAS or isoniazid and PAS was found to decrease markedly the incidence of drug-resistant strains (Medical Research Council, 1950, 1953*c*). The response to combined treatment, measured either in terms of radiological improvement or of sputum conversion, was also slightly superior to the response when streptomycin or isoniazid were used alone.

In considering *in vitro* experiments designed to elucidate the combined activity of PAS with streptomycin or isoniazid two types of experimental systems can be used. In the first of these, sensitive organisms are assumed to be capable of multiplication in tuberculous lesions during treatment so that the effectiveness of the treatment would be limited by the ability of the drugs

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to penetrate in bacteriostatic concentrations to the sites where such multiplication occurred. On this assumption improved results would be expected if the minimal inhibitory concentration of the drugs together was less than that of either drug alone. Vennesland, Ebert & Bloch (1948) found marked enhancement of inhibition when concentrations of either streptomycin or PAS that were moderately inhibitory alone were mixed with a non-inhibitory concentration of the other drug. Furthermore, when multiplying bacilli were subjected to gradually increasing streptomycin concentrations during repeated cultivation in sub-bacteriostatic concentrations of the drug, the addition of very low concentrations of PAS prevented the emergence of resistant strains (Graessle & Pietrowski, 1949; Vennesland *et al.* 1948). When small inocula of a sensitive strain were added to tubes containing serial dilutions of isoniazid, the addition of low concentrations of PAS also prevented the shift in the inhibitory end-point due to the growth of resistant bacilli (Knox, King & Woodroffe, 1952; Aitoff, 1952). The second experimental system assumes that bacteriostatic drug concentrations are present throughout tuberculous lesions soon after the start of treatment. The effectiveness of combined treatment would then depend on the speed with which the initial and usually large population of sensitive bacilli was killed. Reasons for supposing that this approach, in which emphasis is laid on the bactericidal activity of the drugs, is more likely to be correct have been given elsewhere (Mitchison, 1954*b*). An attempt was therefore made to study the influence of PAS on the bactericidal activity of streptomycin or isoniazid and the extent to which it can modify the emergence of drug-resistant bacilli from relatively large sensitive populations.

METHODS

Screw-capped bottles containing 20 ml. of a culture of the H37Rv strain of *Mycobacterium tuberculosis*, grown for 9–10 days in modified Dubos and Davis Tween albumin medium (Medical Research Council, 1953*a*), were centrifuged for 10 min. to remove the larger aggregates of bacilli. The supernatant fluid was removed and stained films from it showed that 80% of the separate bacillary units consisted of single bacilli or pairs of bacilli.

A volume of 0.5 ml. of this supernatant was added to 4.5 ml. of Tween albumin medium (in screw-capped bottles) containing different concentrations of streptomycin, isoniazid or the dihydrate of the sodium salt of PAS (NaPAS). Two concentrations of each of these drugs were used. The higher of these (streptomycin 20 units/ml., isoniazid 2 μ g./ml., NaPAS 100 μ g./ml.) corresponded to the approximate peak serum concentrations found in patients treated with streptomycin at 1 g./day, isoniazid 200 mg./day or NaPAS 20 g./day (Singh & Mitchison, 1954; Nilsson, 1953). The lower concentrations (streptomycin 2 units/ml., isoniazid 0.2 μ g./ml., NaPAS 10 μ g./ml.) were about 4 to 16 times the minimum concentration that inhibits the growth of tubercle bacilli in Tween albumin medium (Medical Research Council, 1953*a*; Mitchison, 1952). A control bottle not containing drug was included in each experiment. The bottles were incubated at 37°. There was no change in the PAS concentration, estimated by the method of Newhouse & Klyne (1949)

during a 3-month period of incubation. At intervals, 0.5 ml. samples were removed from these bottles for viable counts on oleic acid + albumin + agar plates using a calibrated dropping pipette and loop as described by Mitchison (1953). The diluent was Tween albumin medium. The plates were sealed with wax-coated cellulose tape and were incubated for 4 weeks. Colonies were counted with a plate microscope.

Where the presence of resistant organisms was suspected, sensitivity tests to streptomycin, isoniazid and PAS were carried out by the Medical Research Council (1953*a*) methods. In one experiment cultures were injected into guinea-pigs by the intramuscular route.

RESULTS

Bactericidal activity

The first experiment (Fig. 1) was a comparison between the bactericidal activity of streptomycin alone (in concentrations of 2 and 20 units/ml.) and of combinations of streptomycin and PAS. Two combinations were used: one with the low therapeutic concentrations of streptomycin 2 units/ml. and NaPAS 10 $\mu\text{g./ml.}$ and the other with the high therapeutic concentrations of streptomycin 20 units/ml. and NaPAS 100 $\mu\text{g./ml.}$ Controls containing NaPAS 10 $\mu\text{g./ml.}$ and 100 $\mu\text{g./ml.}$ alone were included. Fig. 2 shows a similar experiment in which isoniazid in concentrations of 0.2 or 2 $\mu\text{g./ml.}$ was used in place of streptomycin. In a third similar experiment (Fig. 3) the activity of streptomycin and isoniazid together was compared with the activity of the mixture of streptomycin, isoniazid and PAS.

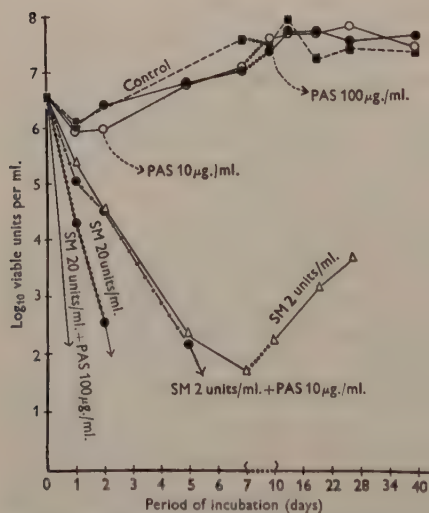


Fig. 1. Bactericidal action of streptomycin alone and in combination with PAS.

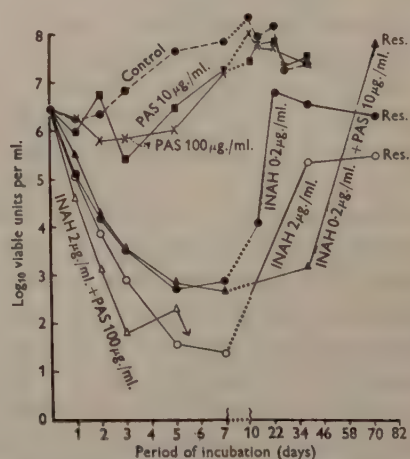


Fig. 2. Bactericidal action of isoniazid alone and in combination with PAS.

In these three experiments PAS alone slightly delayed the growth of the cultures (Figs. 1 and 2) or did not influence them at all (Fig. 3); there was no

significant difference between the activities of 10 and 100 $\mu\text{g.}$ PAS/ml. On the other hand, streptomycin alone, isoniazid alone and the combinations of streptomycin+isoniazid were all actively bactericidal, the activity of the higher concentrations being greater in each case.

It can be seen that NaPAS at 10 $\mu\text{g.}/\text{ml.}$ had no effect on the bactericidal activity of either streptomycin 2 units/ml., isoniazid 0.2 $\mu\text{g.}/\text{ml.}$ or the combination of these concentrations of streptomycin and isoniazid. However, the addition of NaPAS 100 $\mu\text{g.}/\text{ml.}$ slightly increased the activity of the higher concentrations of streptomycin, isoniazid and streptomycin+isoniazid. Thus, as is shown in Fig. 1, growth was obtained for the last time from the culture containing streptomycin 20 units/ml. on the 2nd day of incubation, whereas the culture containing streptomycin 20 units/ml. and PAS 100 $\mu\text{g.}/\text{ml.}$ yielded

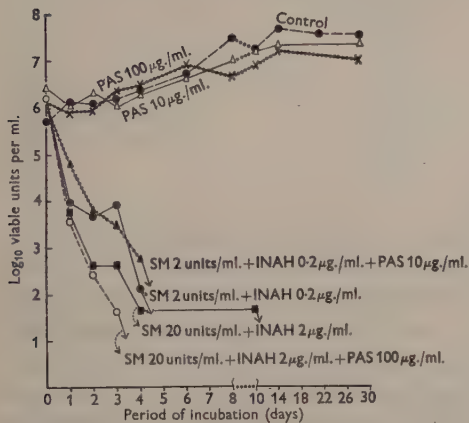


Fig. 3. Bactericidal action of combination of streptomycin, isoniazid and PAS.

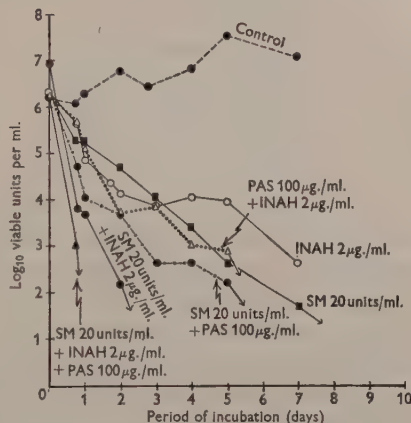


Fig. 4. Bactericidal action of streptomycin and isoniazid in combination with PAS.

no growth when sampled at 1 day. The bactericidal activity of isoniazid at 2 $\mu\text{g.}/\text{ml.}$ was less than that of isoniazid 2 $\mu\text{g.}/\text{ml.}$ + NaPAS 100 $\mu\text{g.}/\text{ml.}$ (Fig. 2) during the first 3 days of incubation, after which no comparison was possible because resistant bacilli grew in the culture containing isoniazid alone. Finally (Fig. 3) the last samples which yielded growth were obtained from the cultures containing streptomycin 20 units/ml. + isoniazid 2 $\mu\text{g.}/\text{ml.}$ on the 10th day, as compared with the 3rd day in the cultures containing these concentrations of streptomycin and isoniazid with the addition of NaPAS 100 $\mu\text{g.}/\text{ml.}$

This increase in the bactericidal activity of streptomycin 20 units/ml., isoniazid 2 $\mu\text{g.}/\text{ml.}$, and their admixture when combined with NaPAS 100 $\mu\text{g.}/\text{ml.}$ was confirmed in a fourth experiment (Fig. 4). Here it was noted that, although cultures were killed more rapidly by streptomycin + PAS than by streptomycin alone, the activity of streptomycin + isoniazid was greater. Thus the apparent synergistic activity of streptomycin and PAS was less than the synergistic activity of streptomycin and isoniazid.

After 28 days of incubation about 2 ml. of each culture from Expt. 4 were injected into guinea-pigs. These animals were sacrificed 10 weeks later and the results of the post-mortem examinations are shown in Table 1, together with the macroscopic appearances of the remainder of the cultures after 3 months of incubation. The culture containing streptomycin produced no lesions in the guinea-pig. However, the animal infected with the culture containing streptomycin + PAS developed tuberculous lesions from which were recovered tubercle bacilli resistant to streptomycin (resistance ratio of 128) but not to

Table 1. *Results of guinea-pig inoculation and appearance, after prolonged incubation, of cultures containing various chemotherapeutic drugs.*

Drugs added to culture	Tuberculous lesions in guinea-pigs	Macroscopic growth after 3 months incubation in culture
Nil	Extensive	+
Streptomycin 20 μ g./ml.	None	—
Streptomycin 20 μ g./ml. } + NaPAS 100 μ g./ml. }	Scanty	—
Isoniazid 2 μ g./ml.	Local lesion	+
Isoniazid 2 μ g./ml. } + NaPAS 100 μ g./ml. }	None	—

PAS. The remainder of this culture failed to show growth after 3 months of incubation. It is probable that very few resistant bacilli capable of growth in 20 units streptomycin/ml. were present in the cultures initially so that sampling variations might account for the failure of the culture containing streptomycin alone to produce lesions in the guinea-pig. The culture containing isoniazid alone eventually showed macroscopic growth of resistant bacilli and it produced local lesions only in the guinea-pig, as might be expected from the low pathogenicity of isoniazid-resistant strains to these animals (Barnett, Bushby & Mitchison, 1953; Middlebrook & Cohn, 1953; Mitchison 1954*a*). A resistant strain did not grow in the culture containing isoniazid + PAS. However, the lack of lesions in the animal infected with this culture should not be interpreted as necessarily meaning that no viable organisms were present, since a small number of isoniazid-resistant bacilli might fail to produce macroscopic disease. From this experiment one can conclude that although PAS may slightly increase the bactericidal activity of streptomycin or isoniazid during the early period when sensitive organisms are being killed, it may be unable to do more than prevent the growth of the resistant bacilli which survive this first phase.

The synergistic action of NaPAS 100 μ g./ml. with streptomycin or isoniazid was found in a further two experiments, making 6 in all. However, it was not invariably reproducible, since it did not occur in part of a seventh experiment.

In sensitivity tests in Tween albumin medium the minimal inhibitory concentration of NaPAS became much lower as the size of the inoculum decreased (Mitchison & Monk, to be published). Where the inoculum of bacilli in these tests resulted in a final concentration of about 10^6 viable units/ml. (the same bacillary concentration as in our bactericidal experiments) the minimal inhibitory

concentration was over 100 μg . NaPAS/ml. On the other hand, where the inoculum was about 10^4 viable units/ml. growth was inhibited by less than 2 μg . NaPAS/ml. It therefore seemed possible that greater synergistic bactericidal activity might be shown if the initial inoculum acted on by the drugs were smaller. No such effect was found. The addition of NaPAS 10 μg ./ml. did not increase the activity of either isoniazid 0.2 μg ./ml. or of streptomycin 2 units/ml., when the initial concentrations of bacilli were between 10^3 and 10^4 viable units/ml.

Drug resistance

In two experiments the viable counts from cultures containing 2 units of streptomycin/ml. alone began to rise after the 1st week and eventually bacilli resistant to streptomycin were obtained. The admixture of NaPAS 10 μg ./ml. with the streptomycin prevented the development of resistant strains in one of these experiments (Fig. 1) since the culture yielded no growth when sampled between the 5th and 40th days of incubation. However, in the other experiment this combination only slowed the growth of streptomycin-resistant bacilli which increased in numbers approximately 100-fold between the 6th and the 44th days. Isoniazid-resistant bacilli also grew rapidly in the culture containing isoniazid 0.2 μg ./ml. alone (Fig. 2). The combination of NaPAS 10 μg ./ml. + isoniazid 0.2 μg ./ml. again only delayed the growth of bacilli resistant to isoniazid and PAS for about a month. Thus one can conclude that NaPAS in the low therapeutic concentration of 10 μg ./ml. may only delay the emergence of resistance.

Resistant strains only appeared in one out of three cultures containing streptomycin 20 units/ml. presumably because of the small number of mutant bacilli in the initial bacterial population which were capable of growth in this drug concentration. However, resistant strains developed in none of the corresponding cultures containing NaPAS 100 μg ./ml. + streptomycin 20 units/ml., neither did one occur in the culture containing NaPAS 100 μg ./ml. + streptomycin 2 units/ml. Furthermore, the addition of NaPAS 100 μg ./ml. to isoniazid 2 μg ./ml. prevented the appearance of isoniazid-resistant bacilli in the experiments shown in Figs. 2 and 4. There is therefore some evidence that resistance is less likely to emerge in cultures containing mixtures of the higher concentrations of PAS and streptomycin than in those with the lower concentrations.

DISCUSSION

The method of measuring the bactericidal activity of the drugs that we have used may be criticized on two grounds. In the first place the addition of PAS might have caused clumping of the bacilli. This seems improbable since, in the cultures containing PAS alone, the viable counts were the same as in the control drug-free cultures after the initial phase of slight inhibition was over. Secondly, carry-over of the drugs from the cultures might have inhibited the growth of surviving bacilli. This possibility was minimized by the small sample (1 loopful) removed from the cultures and by the large area on the plate over

which each sample was spread. Nevertheless, a concentration of NaPAS 100 $\mu\text{g./ml.}$ is about 100 times that necessary to inhibit growth of sensitive bacilli on solid media, so that some of the counts of $<10^3$ viable units/ml. taken from cultures containing 100 $\mu\text{g. NaPAS/ml.}$ may have been diminished by carry-over of this compound.

Bearing in mind these criticisms of the method it remains probable that there was some synergistic bactericidal activity between PAS and isoniazid, PAS and streptomycin and between PAS and streptomycin + isoniazid, when these drugs were present in high concentrations. This synergism was weak and not always reproducible, but it stands in contrast to the antagonistic activity of terramycin (oxytetracycline), which was shown by Mackaness & Smith (1953) to prevent the bactericidal action of isoniazid on tubercle bacilli. Thus although both PAS and terramycin, when acting alone, appear primarily bacteriostatic, their modifications of the actions of such bactericidal drugs as streptomycin and isoniazid are different.

From the clinical point of view any synergistic bactericidal activity of PAS would seem to be too small to influence the response of patients to treatment with streptomycin or isoniazid. Groups of patients treated with either streptomycin + isoniazid, isoniazid + PAS or with streptomycin + isoniazid + PAS, showed the same radiological and bacteriological improvement in a United States Public Health Service Investigation (Ferebee & Mount, 1954). In treatment, the superiority of streptomycin + PAS over streptomycin alone (Medical Research Council, 1950) and the probable superiority of isoniazid + PAS over isoniazid alone (Medical Research Council, 1953*c*) is therefore likely to be due largely to the suppression of drug resistance.

Our finding that combinations of low concentrations of PAS and isoniazid or streptomycin only delayed the emergence of drug-resistant bacilli, whereas combinations of high concentrations suppressed their growth, is consistent with results of the Medical Research Council (1952) trial in which streptomycin-resistant strains were obtained more frequently from patients treated with streptomycin and a low dosage of PAS (5 or 10 g. NaPAS/day), than in those treated with streptomycin and a high dosage of PAS (20 g. NaPAS/day). In contrast the number of isoniazid-resistant strains isolated did not differ significantly when isoniazid was given with 10 g. NaPAS/day or when it was given with 20 g. NaPAS/day (Medical Research Council, 1953*c*). However, the manner in which the doses of PAS were administered differed in these two trials. When given with streptomycin the number of doses during the day was constant, so that the peak serum concentrations following the smaller doses would have been lower. On the other hand, when given with isoniazid the total amount of PAS given in each dose was the same, but the frequency of administration was halved, so the peak serum concentrations during high or low total daily dosage would have been similar. We have no information on the relation between fluctuations in the concentrations of PAS in serum and in the tissues, but it remains possible that the height of peak serum concentrations may be a more important determining factor in preventing drug resistance during combined therapy than the maintenance of a high average concentration.

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A Survey of Inhibitory Compounds for the Separation of Yeasts and Bacteria in Apple Juices and Ciders

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SUMMARY: Selected species of yeasts, lactic acid bacteria and acetic acid bacteria were tested against twenty-six antibiotics and twenty other organic compounds during a search for methods for separating chosen members of the mixed microflora of apple juices. Some of these compounds were found to be specifically inhibitory against either yeasts or lactic acid bacteria, but few inhibited completely such acetic acid bacteria as were tested.

The isolation of yeasts and lactic acid bacteria from apple juices and ciders calls for suitable differential media since the raw materials contain moulds and acetic acid bacteria whose growth must be restricted, otherwise they overgrow the primary isolation plates. In fresh and actively fermenting juices it is also difficult to make an accurate count of the lactic acid bacteria because of the large number of yeasts present, whereas in fully fermented ciders the counting problem is reversed since these lactic acid bacteria then form the major part of the microflora.

In preliminary work it was found that the following measures proved partially successful: (a) the use of apple juice media at pH 4.8 to discourage non-aciduric organisms; (b) anaerobic incubation to encourage the growth of lactic acid bacteria, followed by a further period of aerobic incubation when a yeast count was also required; (c) incorporation of 50 p.p.m. Acti-dione in media when isolating bacteria or 50 p.p.m. aureomycin when yeasts were being examined; (d) use of 100 p.p.m. diphenyl to suppress mould growth.

Certain yeast species resisted Acti-dione, and bacterial colonies occasionally grew in the presence of aureomycin. A search was therefore made for more selective inhibitory compounds which, incorporated in a medium, would permit only the growth of (i) yeasts, (ii) lactic and acetic acid bacteria, (iii) lactic acid bacteria or (iv) individual yeast genera or species of lactic acid bacteria. A simple screening method was used so that a large number of compounds could be tested against a wide range of yeasts and aciduric bacteria chosen from type culture collections. Each compound was tested in a standard malt wort at a number of concentrations to determine at which of these inhibition occurred; the true minimum inhibitory concentration was not determined.

METHODS

Organisms. The organisms used in these tests are given in Table 1. The yeasts were obtained from the Centraalbureau voor Schimmelcultures, Delft, representing at least one species of seventeen genera of non-pathogenic yeasts. With

the exception of *Candida pulcherrima*, all were type strains as given by Lodder & van Rij (1952); the strain of *C. pulcherrima* was that used by van der Walt (1952) in his tests for pigment production.

Both hetero- and homo-fermentative lactic acid bacteria were chosen from the genera *Leuconostoc*, *Streptococcus* and *Lactobacillus* (*Bergey's Manual*, 1948). In addition, two unidentified Gram-positive heterofermentative bacteria were included (both isolated from cider), one a coccus and the other a rod. Apart from these last two organisms and *Acetobacter mobile*, all the bacteria were obtained from the National Collection of Industrial Bacteria, Teddington, Middlesex.

Table 1. *List of test organisms used*

Yeasts	Bacteria
Family ENDOMYCETACEAE	Lactic acid bacteria
1. <i>Schizosaccharomyces pombe</i>	Genus <i>Leuconostoc</i>
2. <i>Saccharomyces cerevisiae</i>	24. <i>L. mesenteroides</i>
3. <i>S. cerevisiae</i> var. <i>ellipsoideus</i>	25. <i>L. dextranicum</i>
4. <i>S. pastorianus</i>	26. <i>L. citrovorum</i>
5. <i>S. rouxii</i>	27. C.1*
6. <i>S. carlsbergensis</i>	Genus <i>Streptococcus</i>
7. <i>S. fragilis</i>	28. <i>S. cremoris</i>
8. <i>Pichia membranaefaciens</i>	Genus <i>Lactobacillus</i>
9. <i>Hansenula anomala</i>	Heterofermenters
10. <i>Debaryomyces hansenii</i>	29. <i>L. brevis</i> var. <i>rudensis</i>
11. <i>Saccharomycoides ludwigii</i>	30. <i>L. brevis</i>
12. <i>Hanseniaspora valbyensis</i>	31. <i>L. fermenti</i>
13. <i>Lipomyces starkeyi</i>	32. <i>L. pastorianus</i>
Family SPOROBOLOMYCETACEAE	33. <i>L. buchneri</i>
14. <i>Bullera alba</i>	34. <i>L. hilgardii</i>
Family CRYPTOCOCCACEAE	35. V822†
15. <i>Cryptococcus laurentii</i>	Homofermenters
16. <i>Torulopsis colliculosa</i>	36. <i>L. casei</i>
17. <i>Brettanomyces bruxellensis</i>	37. <i>L. plantarum</i>
18. <i>Candida utilis</i>	38. <i>L. leichmanni</i>
19. <i>C. mycoderma</i>	<i>Acetobacter</i> spp.
20. <i>C. pulcherrima</i>	39. <i>A. keutingianum</i>
21. <i>Kloeckera apiculata</i>	40. <i>A. pasteurianum</i>
22. <i>Trigonopsis variabilis</i>	41. <i>A. aceti</i>
23. <i>Rhodotorula glutinis</i>	42. <i>A. orleanense</i>
	43. <i>A. mobile</i>

* Heterofermentative coccus isolated from cider.

† Heterofermentative rod isolated from cider.

Medium. The organisms were maintained and tested on a wort medium (pH 5.4) prepared from the same batch of malt by the method of Lodder & van Rij (1952), but sterilized by intermittent steaming instead of autoclaving. Two liquid worts were prepared, one at sp.gr. 1.060 for subculturing yeasts and the other at sp.gr. 1.040 for bacteria. The solid medium for the actual tests was prepared by adding 2% agar to wort of sp.gr. 1.040.

Compounds tested. The compounds used in these tests are listed in Table 2, which also, where necessary, gives the sources from which they were obtained.

Table 2. *Compounds tested for possible inhibitory action against the yeasts and bacteria*

Antibiotics	
Compound	Address of supplier
Compound A18	Glaxo Laboratories Ltd., Sefton Park, Stoke Poges, Buckinghamshire
„ A 228	
„ A 432	
„ A 583	The Upjohn Co., Kalamazoo, Michigan, U.S.A.
Acti-dione	
Actinomycin	
Aspergillie acid	Northern Regional Research Laboratory, Peoria 5, Illinois, U.S.A.
Aureomycin	Lederle Laboratories Division, Aberdare, Glam., S. Wales
Bacitracin	National Institute for Medical Research, Mill Hill, London, N.W. 7
Chloramphenicol	Parke, Davis and Co. Ltd., Hounslow, Middlesex
Clavatin	Biochemical Laboratories, Imperial College, London, S.W. 7
Frequentin	I.C.I. Ltd., Butterwick Laboratories, Welwyn, Hertfordshire
Gliotoxin	Northern Regional Research Laboratory, Peoria 5, Illinois, U.S.A.
Kojic acid	Biochemical Laboratories, Imperial College, London, S.W. 7
Licheniformin	National Institute for Medical Research, Mill Hill, London, N.W. 7
Neomycin	The Upjohn Co., Kalamazoo, Michigan, U.S.A.
Nisin	Applin and Barrett Ltd., Yeovil, Somerset
Polymyxin B	Burroughs Wellcome and Co., Euston Road, London, N.W. 1
Penicillin G	Antibiotics Research Station, Clevedon, Somerset
Streptomycin	
Streptothricin	
Subtilin	Merck and Co. Inc., Rahway, New Jersey, U.S.A.
Terramycin	National Institute for Medical Research, Mill Hill, London, N.W. 7
Thiolutin	I.C.I. Ltd., Butterwick Laboratories, Welwyn, Hertfordshire
	Northern Regional Research Laboratory, Peoria 5, Illinois, U.S.A.
Tomatine	Eastern Regional Research Laboratory, Philadelphia 18, Pa., U.S.A.
Tyrothricin	Sharp & Dohme Ltd., Hoddesdon, Hertfordshire

Other compounds

β -Phenylethyl alcohol; Benzoic acid; Methyl-*p*-hydroxybenzoate; Ethyl-*p*-hydroxybenzoate; Propyl-*p*-hydroxybenzoate
 Dehydroacetic acid; Diphenyl; Ferbam (Ferric dimethyl dithiocarbamate)
 Pandurol, J. M. Collet & Co. Ltd., Bristol Road, Gloucester
 Pentachlorophenol; *p*-Nitrophenol; 2:4-Dinitrophenol; *o*-Phenylphenol; 1:3-Dichloro-2-naphthol
 8-Hydroxyquinoline; Salicylanilide; Sorbic acid; Mercaptoacetic acid; Thiourea.

Method of testing for possible inhibitors

Preparation of inocula. Actively growing cultures (5 ml.) were centrifuged and the organisms resuspended in 0.5 ml. sterile saline.

Preparation of test plates. Stock solutions of the inhibitory compounds were freshly prepared, water-soluble compounds were dissolved in sterile water without further sterilization; others were dissolved in absolute ethanol, acetone or pyridine. For antibiotics, 1.66 ml. of solutions of 5000, 500 and 100 p.p.m. dilutions were added to 15 ml. lots of molten (50°) wort agar in 1 oz.

screw-capped bottles to give final concentrations of 500, 50 and 10 p.p.m. respectively. The contents of the bottles were mixed, poured into sterile Petri dishes, allowed to set and dried at 25° for 2 hr. before inoculation. Similarly, 0.63, 0.46, 0.31 and 0.15 ml. of 50,000 p.p.m. solutions of the other inhibitory compounds were used to give final concentrations of 2000, 1500, 1000 and 500 p.p.m. Occasionally it was necessary to depart from these methods when using less soluble compounds or where intermediate concentrations in the agar were required. Control plates containing the greatest concentration of solvent used in each test were also prepared.

Inoculation of plates. Three complete sets of plates were prepared for each compound, each set being inoculated with one group of the test organisms, i.e. yeasts, acetic acid bacteria or lactic acid bacteria. Individual plates were placed upon a card marked with a numbered grid which acted as a guide for the inoculation pattern. This ensured that every organism was spotted in its correct position on the appropriate set of plates. A 2 mm. wire loop was used for all inoculations.

Records. All plates were incubated at 25° aerobically for yeasts and acetic acid bacteria, but for lactic acid bacteria the plates were kept under partial vacuum in a vessel containing pyrogallol and sodium carbonate (Millis, 1951). The sizes of the colonies were estimated visually after 6 and 12 days of incubation; only the later observations are recorded in this paper. When the lowest concentration used for any compound entirely inhibited one or more groups of organisms, tests were repeated at still lower concentrations.

RESULTS

Since it is not possible to present in detail here the effect of each compound on every organism over the whole range of concentrations tested, a copy of the complete results will be deposited with the Librarian, General Library, British Museum (Natural History), London S.W. 7, for reference purposes.

The compounds are classified under three headings according to their action on the different organisms; all concentrations are given in p.p.m., unless stated otherwise. When combinations of compounds are given it is probable that the effective concentrations of the constituents would be lower in practice since the values quoted relate to their effect when used singly.

Compounds suitable for selective media

These compounds differentially inhibited one or more groups of the organisms shown in Table 1.

Inhibition of bacteria. All the bacteria tested were inhibited by the following compounds at the stated concentrations while leaving the yeasts unaffected: aureomycin, 500; chloramphenicol, 500; pentachlorophenol, 10; actinomycin, 0.5 plus aureomycin, 50; penicillin G, 500 plus aureomycin, 50; terramycin, 500 plus aureomycin, 50.

Lactobacillus brevis, *L. brevis* var. *rudensis* and *L. buchneri* were the most resistant species of lactobacilli and were inhibited only at the highest concentrations of aureomycin, chloramphenicol or pentachlorophenol. Actino-

mycin inhibited the lactobacilli at very low concentrations, but actinomycin and penicillin G had no effect on the acetic acid bacteria and inhibited both groups of organisms only when combined with 50 p.p.m. of aureomycin. The mixture tyrothricin, 500 plus aureomycin, 50 also inhibited the yeasts *Debaryomyces hansenii*, *Lipomyces starkeyi*, *Cryptococcus laurentii* and *Kloeckera apiculata*; this mixture is therefore only suitable if these are either absent from the sample or need not be isolated.

Inhibition of yeasts. All the yeasts were inhibited by the following compounds, which did not affect the bacteria: compound A, 228, 500 units/ml.; methyl-*p*-hydroxybenzoate, 1000; ethyl-*p*-hydroxybenzoate, 1000; dehydroacetic acid, 250; Ferbam, 50; Acti-dione, 125 plus gliotoxin, 50; 8-hydroxyquinoline, 250 plus Acti-dione, 10; thiolutin, 250 plus Acti-dione, 10; *p*-nitrophenol, 250.

In general, the film-forming yeasts were very resistant to most of the inhibitory compounds. *Saccharomyces cerevisiae* var. *ellipsoideus* was the most resistant of the fermenting yeasts. This property might prove useful as a supplementary test to differentiate it from its less resistant parent species, if other strains of the two yeasts reacted in a similar manner to the test organisms. *Lipomyces starkeyi*, *Kloeckera apiculata* and *Rhodotorula glutinis* were very sensitive to most of the compounds yet, surprisingly, resisted the action of Acti-dione alone.

Acti-dione and gliotoxin had a complementary inhibitory action. The suggested concentration of Acti-dione in this combination may be reduced to 50 p.p.m. when *Cryptococcus laurentii* is known to be absent from the sample.

Isolation of lactic acid bacteria. The specific isolation of lactic acid bacteria proved most difficult because many compounds which prevented the growth of acetic acid bacteria also inhibited the lactic acid bacteria at the same or lower concentrations. Only sorbic acid at 2000 p.p.m. inhibited the acetic acid bacteria without affecting the lactic acid bacteria. Sorbic acid did not inhibit all the yeasts, and in order to prevent yeast growth completely this compound had to be used in admixture with dehydroacetic acid, 250; or gliotoxin, 500; or Acti-dione, 50. The last mixture may still permit the growth of *Brettanomyces bruxellensis* and *Trigonopsis variabilis*, but as these are encountered only infrequently this may be of little consequence.

Compounds of limited value for selective media

Few raw materials would contain all the yeasts and bacteria used in these tests, so that the compounds in this section may be equally valuable for isolating members of a more limited microflora.

Resistant yeasts. Each of the inhibitory compounds listed below allowed the selective isolation of a restricted number of yeasts which are coded as in Table 1. Bacterial contaminants may, if necessary, be inhibited by the addition of one of the compounds which inhibited all the bacteria tested (see above paragraphs labelled 'Inhibition of bacteria').

In the tabulated records which follow, the data are given in the following

way: compound, concentration used in p.p.m., code number (from Table 1) of resistant organisms.

Compound A. 228, 500: 7, 8, 9, 18, 19, 22.	Clavatin, 500: 3, 22.
Acti-dione, 500: 7, 12, 13, 17, 21, 22, 23.	Frequentin, 125: 8, 9, 19.
Acti-dione, 50 + gliotoxin, 50: 15.	Frequentin, 250: 19.
Acti-dione, 50 + sorbic acid, 2000: 17, 22.	Gliotoxin, 500: 8, 9, 11, 19.
Aspergillie acid, 500 or 8-hydroxy-quinoline, 250: 9.	Phenol, 2000: 3, 7, 8, 9, 17, 18.
<i>n</i> -propyl- <i>p</i> -hydroxy-benzoate, 250: 3, 7, 9, 10, 15, 18.	Polymyxin B, 500: 1, 3, 5, 7, 8, 9, 22.
	Salicylanilide, 1500: 3, 4, 12, 17.
	Sorbic acid, 1000: 2, 3, 5, 6, 16, 17, 18, 22.
	Thiolutin, 250: 8, 19.

Resistant lactic acid bacteria. The compounds given below allowed the preferential isolation of certain of the lactic acid bacteria (code numbers as in Table 1). Certain of these compounds will also suppress yeasts and acetic acid bacteria present as contaminants. Where they are ineffective in this respect they may be combined with a compound from among those listed above in the paragraph *Isolation of lactic acid bacteria*.

Aurcomycin, > 50 and < 500: 24, 29, 30, 33.	2:4-Dinitrophenol, 100: 29, 30, 31, 32, 33, 34, 35, 36, 37, 38.
Benzoic acid, > 2000: 33.	<i>o</i> -Phenylphenol, 250: 24, 26, 29 to 35, 37, 38.
Gliotoxin, 500: 33.	8-Hydroxyquinoline, 2000: 25, 29, 30, 36, 37.
<i>n</i> -Propyl- <i>p</i> -hydroxy-benzoate, 1000: 29, 30, 31, 32, 33, 35, 36, 38.	Salicylanilide, > 2000: 33, 36.
Chloramphenicol, 500: 27, 33, 36, 37.	Streptomycin, 500: 27, 29, 30, 31, 33, 37.
Clavatin, 500: 29, 30, 33.	Mercaptoacetic acid, 200: 31, 32, 34, 37.
Neomycin, > 500: 34, 36, 37.	Thiolutin, > 250: 29.
Panduro, 10: 29, 30, 31, 32, 33, 35, 38.	

But for the fact that 250 p.p.m. *o*-phenylphenol inhibited *Leuconostoc mesenteroides*, C. 1, *Streptococcus cremoris* and *Lactobacillus casei*, this compound could have been included among those listed above as useful for the isolation of lactic acid bacteria since it inhibited all the yeasts and acetic acid bacteria at this concentration.

The heterofermentative members of *Lactobacillus*, e.g. *L. buchneri*, were more resistant than the homofermentative species to the action of the test compounds. Similarly, the heterofermentative *Leuconostoc* spp. were less easily suppressed than the homofermentative *Streptococcus cremoris*, but the resistance of the cocci as a group was less than that of the rods.

Compounds useless for selective media

The remaining substances tested, namely, compounds A 18 and A 583, bacitracin, kojic acid, licheniformin, nisin, β -phenylethyl alcohol, streptothricin, subtilin, thiourea and tomatine, were unsuitable for preparing differential media since they were not sufficiently selective in their action even at the highest concentrations used in these tests.

Compounds suitable for suppressing moulds

A number of the compounds tested are normally used as fungicides, but of these only diphenyl was found suitable for incorporation in media for the suppression of mould contaminants. When used at 2000 p.p.m. the lactic acid and acetic acid bacteria were unaffected, and the only yeasts inhibited were *Debaryomyces hansenii*, *Bullera alba*, *Cryptococcus laurentii*, *Kloeckera apiculata* and *Rhodotorula glutinis*. In routine practice diphenyl was used at 100 p.p.m. and all the yeasts grew satisfactorily at this concentration while mould contaminants were suppressed.

Effect of solvents

Of the inhibitory compounds tested twenty-five were soluble in water, eighteen in ethanol, two in dilute pyridine water and one in acetone. The effects of these solvents on the test organisms were as follows.

Yeasts. The control plates contained 10% (v/v) ethanol, the highest concentration used in the actual tests. When the experiments were begun it was found that seven of the yeasts (*Debaryomyces hansenii*, *Hanseniaspora valbyensis*, *Lipomyces starkeyi*, *Bullera alba*, *Cryptococcus laurentii*, *Kloeckera apiculata* and *Rhodotorula glutinis*) sometimes failed to grow or the colonies were much decreased in size compared with those on the normal control plates. This was largely overcome by using inocula not more than 3 days old, preparing fresh media every 14 days and standardizing the period of plate-drying before inoculation. Under these conditions only the ethanol sensitive yeasts, *Bullera alba*, *Kloeckera apiculata* and *Rhodotorula glutinis* failed to develop fully. For these organisms no results are recorded in cases when it was found that a particular concentration of ethanol was reinforcing the inhibitory effect of a given compound.

The initial rate of growth of the other yeasts was slightly delayed by ethanol, but after 12 days incubation, the colonies were the same size as those on the normal control plates. Similar results were obtained with acetone and pyridine water.

Bacteria. The lactic acid bacteria were not affected by any of the four solvents, while the growth of the acetic acid bacteria was slightly retarded on the ethanol control plates during the early stages of incubation.

DISCUSSION

These results show that some of the compounds tested are suitable for the selective isolation of yeasts and lactic acid bacteria from mixed microfloras such as are found in apple juices and ciders. Similar differential isolation techniques have been used in other fermentation industries, although a smaller number of inhibitory compounds have been tested (Strandskov, Brescia & Bockelmann, 1953; Gray & Kazin, 1946). One of the main experimental uses of antibiotics in such instances has been the suppression of undesirable organisms in several fermentation products (Day, Serjak, Stratton & Stone, 1954; Strandskov & Bockelmann, 1953; Strandskov, Baker & Bockelmann,

1953). Much smaller amounts of inhibitory compounds were required to suppress such species under these conditions than were needed in pure culture methods, no doubt because of the lack of competition for nutrients in the latter. A wide range of compounds was tested by Fitzgerald & Jordan (1953) for the suppression of pure cultures of oral lactobacilli; the inhibitory levels quoted agree closely with those presented here. The compounds of the greatest interest in the present tests are summarized below.

Acti-dione. Whiffen (1948) found that this substance had no effect on bacteria but inhibited many, although not all, yeast genera. Similar results were obtained in the present tests and in the routine plating of apple juice samples on media containing Acti-dione. Acti-dione has been used for counting bacterial contaminants in brewery yeasts and beers (Phillips & Hanel, 1950; Green & Gray, 1951; Strandkov & Bockelmann, 1951); it was found ideal for this purpose because brewery yeasts are especially sensitive to it.

Actinomycin was the most effective inhibitor for the lactic acid bacteria; according to Reilly, Schatz & Waksman (1945) it is also fungistatic.

Aureomycin was valuable for inhibiting bacterial growth, especially acetic acid bacteria; it had no effect on yeast growth at 500 p.p.m. (Hesseltine, Hauck, Hagen & Bohonos, 1952). Terramycin, which has a similar chemical constitution, was less effective.

Chloramphenicol, often used to inhibit Gram-negative bacteria, did not suppress the acetic acid bacteria differentially.

Dehydroacetic acid was a good yeast inhibitor at 250 p.p.m. without affecting bacterial growth. It is only fungicidal at higher concentrations (Eeckhaut, 1952; Mossel & de Bruin, 1950; Wolf, 1950).

Diphenyl has been used for several years at Long Ashton to prevent mould growth (Hertz & Levine, 1942). At 100 p.p.m. it does not affect yeast or bacterial growth, in contrast with many other fungicides which suppress these organisms at fungistatic concentrations.

Frequentin was found to be an effective yeast inhibitor; its use for this purpose has not been previously reported. It is also fungicidal (Curtis, Hemming & Smith, 1951).

Gliotoxin inhibited most of the test yeasts at 500 p.p.m. but was not as selective as frequentin since it was also anti-bacterial at this level.

8-Hydroxyquinoline was a most effective yeast inhibitor at concentrations which left the test bacteria unaffected. Previous reports have concerned its effect on fungi and bacteria (Albert, 1953).

Sorbic acid was the only compound that inhibited the acetic acid bacteria but not the lactobacilli (Emard & Vaughn, 1952). It also inhibited many of the test yeasts. Phillips & Mundt (1950) used this compound to inhibit film yeasts in pickle brines to allow the preferential growth of species of *Leuconostoc* and *Lactobacillus*.

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A Study of Organisms of the Pleuropneumonia Group by Electron Microscopy

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SUMMARY: Electron micrographs of organisms of the pleuropneumonia group are presented, which are consistent with a simple form of development that starts from a small dense body. This grows into a larger particle which may be round, oblong or filamentous in the liquid medium. Later, circumferential concentrations and central thinning occur. Eventually, the concentrations become the dense bodies from which new forms develop. A cell wall was not demonstrable.

As pointed out in a recent review (Klieneberger-Nobel, 1954), morphological descriptions of the organisms of the pleuropneumonia group vary considerably, and the interpretations of the pictures observed have caused a great deal of controversy. The electron microscope has so far proved to be of little assistance in this field, but the examination by this means of organisms with small filterable forms should offer great advantages over light microscopical methods.

METHODS

The agalactia strain used was isolated from the milk of a diseased goat. The milk had been sent to London from Rome. The goat pleuropneumonia strain was sent from Ankara. The purulent material was taken *post mortem* from a bronchiectatic rat which had been operated upon and sent to E. K-N. by Dr Cheng (see Cheng, 1954).

The organisms were grown either in meat infusion peptone broth or in 'boiled blood broth' (Klieneberger-Nobel, 1954), to which was added sterile horse serum heated at 56° for 1 hr. At first, the serum was filtered through Seitz pads to sterilize it but the filtrate contained asbestos fibres that appeared in the electron micrographs, and Gradocol membranes were subsequently used for filtration. The cultures were spun for 1 hr. in an angle centrifuge at 4000 r.p.m. Preliminary dispersion was carried out in a tissue grinder when clumps were visible. Sometimes the suspensions were filtered through a sintered glass filter (G 5 over 3, Schott and Genossen, Jena) before centrifugation. The supernatant was discarded after spinning, and the pellet was suspended in a Tyrode-Ringer solution of the following constitution: sodium chloride, 8.0 g.; potassium chloride, 0.2 g.; calcium chloride, 0.2 g.; glucose, 1.0 g.; sodium phosphate (NaH_2PO_4), 0.05 g.; sodium bicarbonate, 1.0 g.; glass distilled water, 1 l. All substances used were of Analar grade.

The Tyrode-Ringer solution was sterilized by Gradocol membrane filtration. It was stored in the cold and freshly made up at frequent intervals. This solu-

tion proved to be a very good suspending fluid. Titration of such suspensions in serum broth and incubation for a period of 10 days showed that the organisms remained viable in it for several hours; the titre was as high as in a similar set of suspensions in serum broth made directly from the original culture. The organisms dispersed well in the fluid and remained dispersed for a considerable time. Sometimes the suspensions were washed in the Tyrode-Ringer solution; but it usually sufficed to resuspend the pellet in 3-4 times the original volume of the culture medium to prevent impurities from appearing in the electron micrographs.

Little success attended our efforts to fix and mount the material in a form suitable for electron microscopy until we adopted a special method devised by one of us (Cuckow, 1955) for other work. In this method, the organisms are fixed progressively by diffusion of fixative while the suspending fluid remains otherwise unchanged. After fixation, the salts in the suspending fluid are gradually removed so that the final preparation may be dried leaving fixed organisms free from obscuring salt crystals.

It will be noted that progressive fixation by diffusion of these organisms had already been shown by one of us (E. K-N.) to be the method of choice ('agar fixation technique'), and the present technique combines the advantages of such progressive fixation with minimum disturbance of electrolyte balance.

For this purpose, a 'formvar' membrane was floated on the Tyrode-Ringer solution to which formaldehyde had been added. Small droplets of the appropriately diluted suspensions were placed on the upper surface of the membrane by means of a finely drawn out capillary pipette. After 3 min. fixation by diffusion of the formaldehyde, the membrane was refloated on distilled water for washing by diffusion through the membrane and remained there for at least 30 min. Then the parts of the membrane carrying the droplets were laid on grids, excess fluid was withdrawn and the grids were dried in the air. It later proved sufficient to fix by diffusion of the formaldehyde direct from concentrated aqueous solution instead of from solution in Tyrode-Ringer medium as suggested above, and all the results shown here have, in fact, been obtained in this rather less refined manner. Washing by diffusion through the membrane has been carried out in all cases.

The specimens were shadowed with gold-palladium alloy at an angle of approximately 30° and the micrographs were taken with a Philips electron microscope with an electron beam of 60 kV.

For comparative morphological studies unfixed suspensions were also examined in the darkground microscope, and fixed and washed membrane preparations were mounted on cover-slips instead of on grids, air-dried and stained in Giemsa solution. Eventually they were dehydrated and mounted in Canada balsam as described by Klieneberger & Smiles (1942).

DESCRIPTION OF THE ELECTRON MICROGRAPHS

Pl. 1, fig. 1, represents particles from an unfiltered young serum broth culture of the organism of agalactia of sheep and goats (incubation 17 hr.). It contains small, roundish, oval and rod-shaped forms of an even opacity. In some of the

bodies a less opaque part is to be seen, which in the round forms appears as a central indentation or depression and in the rod-like and filamentous forms occupies a zone lying centrally and along their minor axes.

The preparations illustrated in Pl. 1, figs. 2 and 3, were made from a sintered-glass filtered 4-day old serum broth culture of the organism of agalactia. Most of the elements have a definite depressed or less opaque part in the middle, no matter whether they are round, rod-like or filamentous.

Pl. 1, fig. 4, shows elements of an unfiltered, 4-day old serum broth culture of agalactia. Slightly bigger and more filamentous forms are present. The central less opaque part is fairly large in the bigger elements. Several bodies are to be seen in which the peripheral material appears to be disposed in denser small bodies. Two dense bodies are seen in the rods (one at either end) and three or more in the roundish forms. The appearances strongly suggest that sprouting occurs from these sites.

Pl. 2, figs. 5-7, show a very variegated picture of the filamentous growth in a 24 hr. serum broth culture of the goat pleuropneumonia organism, which was not yet visibly turbid. The culture was spun and the small pellet resuspended in the Tyrode-Ringer solution. The differentiation into opaque and relatively transparent zones can be seen everywhere, and the appearances suggest that growth continues from the granular points of concentration.

Pl. 2, fig. 8, shows the elements contained in the caseous pus from a lung abscess of a rat affected by bronchiectasis. This widespread lung infection of laboratory rats is caused by an organism of the pleuropneumonia group which was called 'L3' when first described (Klieneberger & Steabben, 1937, 1940). Although several hundreds of rats were studied, it was never possible, by light microscopical methods, to demonstrate the particles of the organism directly in the lesions, though they invariably grew in innumerable small colonies on the medium used. By the electron microscope this gap has now been closed. The pus was ground in a small amount of the Tyrode-Ringer solution, and by fractional centrifugation the pus and tissue cells were eliminated and a very slightly turbid liquid was obtained which contained a large number of elements closely resembling the bodies of agalactia and pleuropneumonia as shown in our illustrations. On the left-hand side of fig. 8, a round element with filamentous form attached is seen which contains areas of two different opacities. In other micrographs the equally characteristic forms with central area of diminished opacity were found.

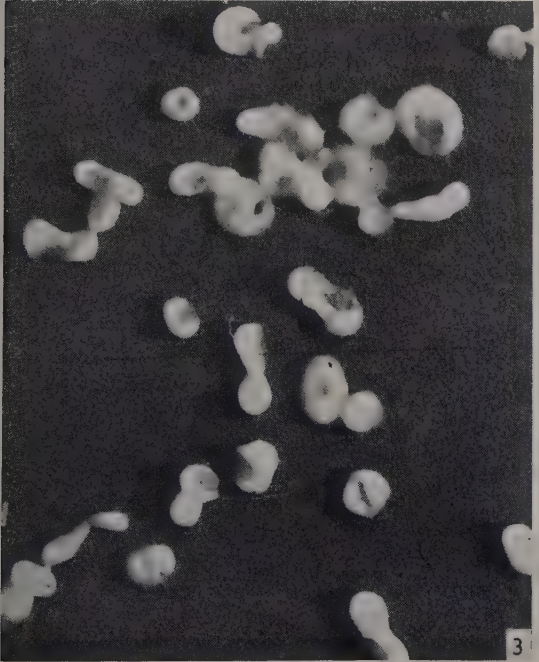
DISCUSSION

When the electron micrographs are compared with micrographs produced by light optical methods such as darkground and staining techniques, it will be seen that all these methods are in very good agreement. However, in the electron micrographs, the organisms are much more distinct and far better differentiated; moreover, the shadowing technique also gives some indication of their thickness. What appears as a granule in the dark-field and a dot in the stained preparation is a dense opaque body in the electron micrograph and may here be round or oval. The ring form so often mentioned and demonstrated by

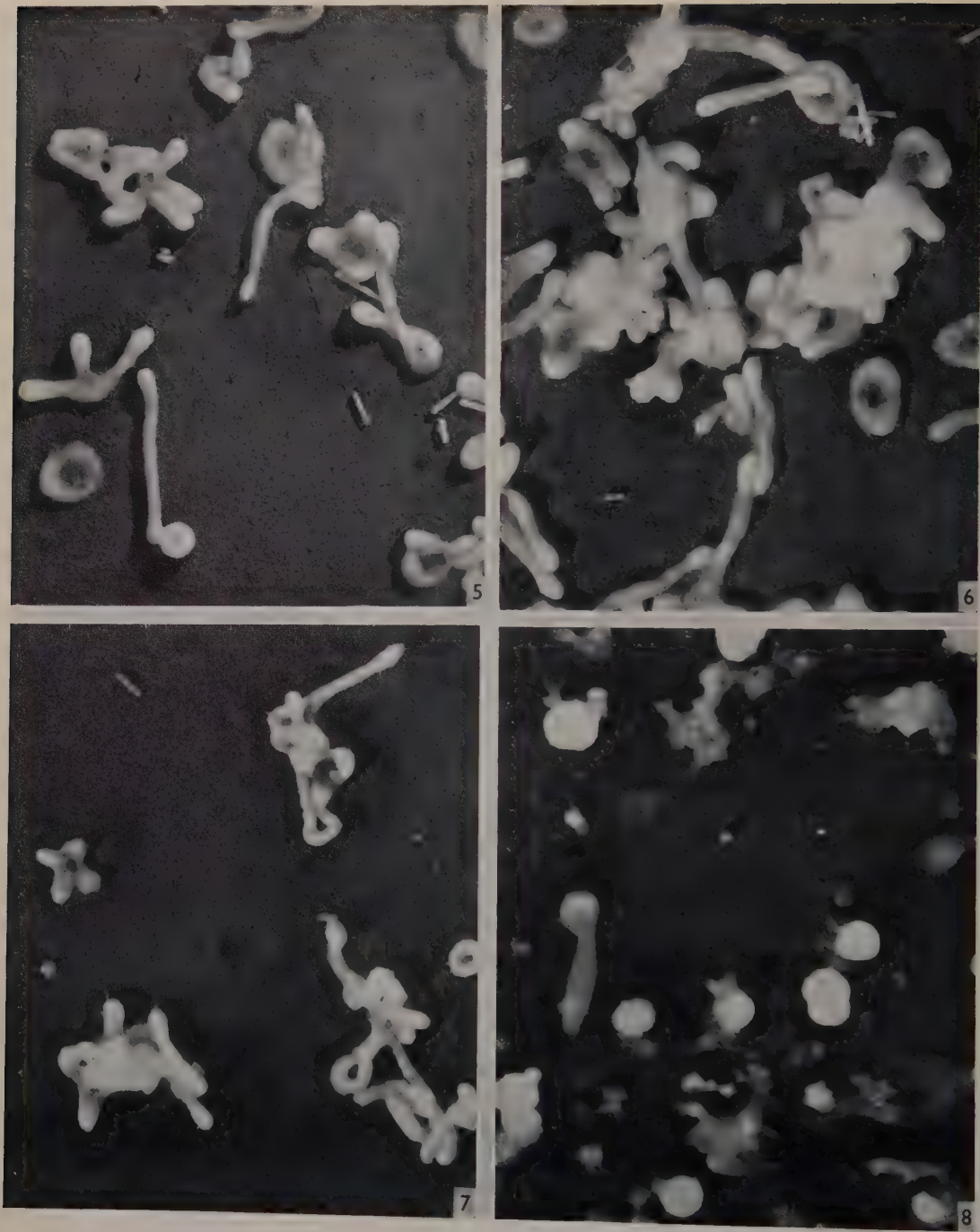
earlier authors in light microscope studies can be correlated with the slightly larger body with a central less opaque part as seen in the electron micrographs. The so-called granules which are occasionally but rarely seen in the dark-field and which in stained preparations have been shown by Klieneberger & Smiles (1942) to lie within the circumference of the forms, can be correlated with the small dense bodies into which in the electron micrographs the dense peripheral material seems to have contracted. The filamentous forms particularly demonstrated by Turner (1935), Tang, Wei, McWhirter & Edgar (1935) and by Ørskov (1942) in the light microscope and recently by Freundt (1952) on the electron microscope show in our electron micrographs as less opaque zones and small dense bodies at their tips. This confirms the view of one of us (E. K-N.) (1954) that they are similar to the round and oval bodies and undergo a similar development; a development that culminates in the formation of the small dense bodies which are the sites of further growth.

From the study of the electron micrographs here presented it is concluded that the development of these organisms is as follows. The initial forms or 'minimal reproductive units', which are usually round (spheres or disks), or oblong or rod-like, consist of uniformly dense material. Soon a differentiation takes place; zones of concentration are formed at the edges, and the centre portions become more transparent. Eventually, the circumferential concentrations become small dense bodies. A rod-like element usually produces two small dense bodies, one at each end. Other bodies produce two, three or more small dense bodies at the circumference. These small dense bodies grow again into the same round, oval or filamentous elements, which may either remain in connexion with the mother element or be set free. By this process of multiplication and growth, all the various configurations described and depicted in the literature and shown in the present illustrations may arise. Outstanding because of their fine quality are the dark-ground micrographs of the organism of bovine pleuropneumonia published by Turner (1935) which show the circumferential concentrations, granule formation and new outgrowth as well as they can be demonstrated by the dark-ground method. However, as Klieneberger & Smiles (1942) pointed out, Turner's interpretations are highly speculative. It seems unnecessary to invent, as he has done, five different 'genethodes' to explain the organisms' apparent pleomorphism. The flexibility of the elements, together with their lack of a rigid cell wall, are a sufficient explanation of this property; the formation of small dense bodies in the way here described and their further development in the same culture explains the variegated appearance of the whole growth. This conclusion was drawn by Klieneberger & Smiles (1942) from their micrographs of stained preparations (see their figs. 3-5 and 7), and has been confirmed by electron micrographs, the resolution of which far surpasses that of photomicrographs.

We wish to thank Dr K. K. Cheng for his kindness in sending the bronchiectatic rat to E. K-N.



E. KLIENEGER-NOBEL AND F. W. CUCKOW—PLEUROPNEUMONIA ORGANISMS. PLATE 1



E. KLIENEBERGER-NOBEL AND F. W. CUCKOW—PLEUROPNEUMONIA ORGANISMS. PLATE 2

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EXPLANATION OF PLATES

All preparations were shadowed with gold-palladium before photographing.

PLATE 1

- Fig. 1. 17 hr. serum broth culture of agalactia. $\times 14,000$.
Figs. 2, 3. Filtered 4-day serum broth culture of agalactia. $\times 14,000$.
Fig. 4. Unfiltered 4-day serum broth culture of agalactia. $\times 7,000$.

PLATE 2

- Figs. 5-7. 24 hr. serum broth culture of organism of goat pleuropneumonia. $\times 14,000$.
Fig. 8. Pus from lung abscess of rat. $\times 14,000$.

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The Site of Action of Penicillin: some Properties of the Penicillin-binding Component of *Staphylococcus aureus*

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SUMMARY: The penicillin-binding component (PBC) of *Staphylococcus aureus* is rapidly inactivated by acid but is more stable at neutral or alkaline pH values. At 2° various preparations lost 25-50 % activity overnight at the optimum pH. Cell-free preparations of PBC (penicillin-binding cell walls or 'lipid particles') showed an initial increase in the amount of PBC available to penicillin. PBC was heat-labile, being completely destroyed by 5 min. at 50°. The final amount of penicillin bound did not vary over the range pH 4.4-7.6, but the rate of binding was somewhat greater at the lower end of this range. No separation of PBC from lipid particles was achieved by several mild techniques. Organic solvents which did not remove lipid material from intact cells also failed to affect PBC; more drastic solvent procedures which were effective in removing lipid appeared to destroy PBC. Intact cells or lipid particles from a penicillin-resistant yeast did not bind penicillin.

The most likely interpretation of the experimental finding (Rowley, Cooper, Roberts & Lester Smith, 1950) that penicillin-sensitive cells were able to bind a small definite amount of penicillin in a specific and irreversible manner was that they contained a small amount of a chemical entity which was termed the penicillin-binding component (PBC). It was thought that PBC was likely to be the initial site of action of penicillin, although direct proof of this could not be afforded. PBC might exist in the cell as an easily removable co-factor or it might be an integral part of a large molecule, and its isolation would then involve the difficult task of separating a very small amount of material from a large amount of similarly constituted impurities. Alternatively, it is theoretically possible that PBC cannot exist separately, but is rather a steric effect produced by the conjunction of two large molecules between which the penicillin happens to fit. Any attempt to separate the two large molecules would then lead to the disappearance of PBC. Nevertheless, it seemed worth while to attempt the preparation of PBC in a soluble form from which chemical purification could begin. The results given below describe some properties of PBC and the lipid-containing particulate fraction in which it is concentrated (Cooper, 1954), and illustrate some of the difficulties encountered in obtaining PBC in soluble form.

METHODS

Uptake of radiopenicillin by intact cells was measured by the method described by Rowley *et al.* (1950), and the preparation of, and measurement of uptake by, penicillin-binding cell walls were as described by Few, Cooper & Rowley (1952). Methods of growing the staphylococcus used, preparation of 'lipid particles' and measurement of penicillin-binding by lipid particles by

use of radiopenicillin and by bioassay were described by Cooper (1954). The radiopenicillin purity was checked by methods described by Cooper, Clowes & Rowley (1954).

RESULTS

Stability of penicillin-binding component

It was important to know to which experimental conditions PBC could be submitted without undue loss. The results illustrated in Fig. 1 show that PBC could be kept at neutral or alkaline pH values for several hours at room temperature even in the cell-free state, but was rapidly and irreversibly inactivated by acid. About 25% of the PBC was lost from intact cells when kept

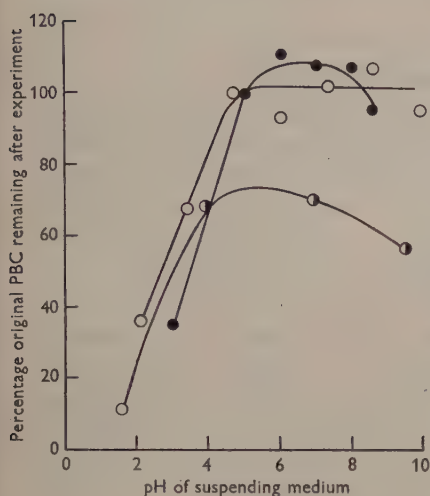


Fig. 1

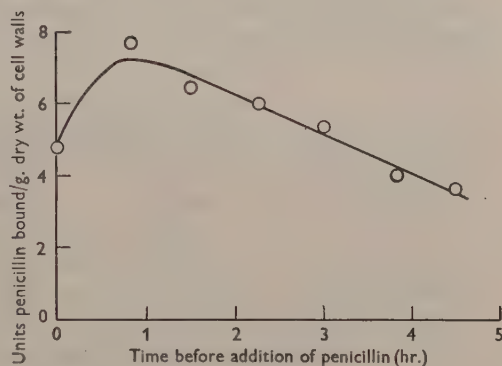


Fig. 2

Fig. 1. Effect of pH value on stability of PBC in 0.1 M-phosphate + 0.1 M-acetate. Assays of the initial activity were made separately in each case. ○—○, intact cells, left for 16 hr. at various pH values and 2°, washed 3 times and adjusted to pH 7 before addition of radiopenicillin. ●—●, cell walls prepared in formalin mixture left for 45 min. at various pH values and 24°, washed twice and adjusted to pH 7 before addition of radiopenicillin. ○—○, unwashed lipid particles, left for 90 min. at various pH values and 24°, neutralized by addition of penicillin in an equal vol. 2 M-phosphate buffer at pH 7.5; binding determined biologically by cup-plate assay of residual unbound penicillin.

Fig. 2. Effect of time of standing in distilled water at 18° on the penicillin-binding capacity of cell walls prepared in formalin mixture. Radiopenicillin was added at various times, and the suspensions then kept in the cold until the end of the experiment, when all were centrifuged and washed together for ³⁵S assay.

overnight at 2° at the optimum pH value (pH 6). In one sample of lipid particles stored overnight at 2° in the presence of cell cytoplasm about 50% of the initial PBC was lost. The curious suggestion (in Fig. 1) that the penicillin-binding ability of cell walls prepared in formalin (Few *et al.* 1952) had increased slightly during the course of the experiment was confirmed in several experiments; it always occurred during the 1–2 hr. following the completion of the formalin cell-wall preparation and then fell fairly rapidly (Fig. 2). A similar

increase in available PBC but without subsequent rapid loss also occurred with lipid particles which had been separated from the cytoplasmic supernatant; it proceeded at about the same rate at 0° as at 37° (Tables 1 and 2). This, with the lack of inhibition of the effect by formalin pretreatment, suggested that the increase was not enzymic. Approximately twice as much PBC is liberated on cell rupture as is available to the penicillin in the intact cell (Cooper, 1954). The final uptake reached in Table 1 corresponds closely with the uptake by lipid particles when inactivation of PBC during rupture is minimized by shaking in the presence of radiopenicillin. The ability of the lipid particles to regain their penicillin-binding capacity on standing suggests that this inactivation may be reversible.

Table 1. *Stability of PBC in unwashed lipid particle suspensions in distilled water at 0° and 37°*

At the beginning of the experiment about 2 hr. had elapsed from start of cell rupture. Penicillin was added (0.07 unit/ml.) at various times and the suspensions then kept at 0° until the end of the experiment. The residual penicillin concentrations were determined by bioassay.

Time of observation (hr.)	Amount of penicillin bound by particle suspension (unit/ml.)		Penicillin bound by lipid particles (unit/g. dry wt. particles)	
	0°	37°	0°	37°
0	0.008	0.008	6.4	6.4
1½	0.031	0.041	25	33
2	0.036	0.028	29	22
3	0.040	0.032	32	25

Table 2. *Effect of temperature on stability of PBC*

Experimental method as in Table 1.

Initially	Amount of penicillin bound by lipid particle suspension (unit/ml.)							
	After 3½ hr. at			After 5 min. at				
	0°	24°	37°	40°	50°	56°	60°	90°
0.031	0.021	0.018	0.014	—	—	< 0.003	—	< 0.003
0.015	—	—	—	0.011	< 0.004	—	< 0.004	—

The apparent difference in rates of loss of PBC from penicillin-binding cell walls and from lipid particles is probably due to the several washes of the former involved in the assay of bound penicillin. Thus PBC may become progressively more easily washed away from cell walls on standing.

PBC was unstable at higher temperatures, none being detectable after 5 min. at 50° (Table 2). Freeze-drying had no effect on the PBC content of intact cells, lipid particles or formalin-prepared cell walls. The PBC-radiopenicillin complex remained attached to intact cells in aqueous suspension very much longer than PBC itself was detectable.

Effect of pH value on penicillin uptake

Fig. 3 shows that the final amount of penicillin bound by resting cells (20 min. total contact) was only a little affected between pH 4.4 and 7.6. Very similar curves were obtained when the penicillin solutions were adjusted to the various pH values for the same period of time and neutralized before addition of cells, showing that the higher uptakes at pH 3 and 4.4 were entirely due to radio-active decomposition products of penicillin. The lowest pH value at which instability of penicillin is small over the course of the experiment is about pH 4.4 (Brodersen, 1947), increasing greatly at lower pH values. However, although the final amount of uptake of radiopenicillin is independent of pH, the rate at which this value is attained is decreased with increase in pH value (Fig. 4). The effect of pH value on the rate of penicillin uptake is of interest in view of reports (Abraham & Duthie, 1946; Eagle, Levy & Fleischman, 1952) which indicated that penicillin is considerably more active at pH 6 than at pH 7.5. Since the minimal inhibitory titre may be the result of a balance between the rate of inactivation of PBC by penicillin and the rate of PBC resynthesis (Maass & Johnson, 1949*b*), any increase in the rate of inactivation of PBC caused by pH changes should result in this balance being struck at lower titres, since the rates of penicillin-binding at pH 5.5 and 7.3 are roughly proportional to penicillin concentration (Fig. 5). It can be calculated from Figs. 4 and 5 that penicillin at 0.025 unit/ml. and pH 7.5 should inactivate PBC as fast as at 0.017 unit/ml. and pH 6.0. The difference between these theoretical minimal inhibition concentrations is rather less than the two- or fourfold differences observed by Abraham & Duthie and by Eagle and colleagues over this pH range, so that changes in penicillin binding-rate are probably not the only effects of pH value on the lethal process caused by penicillin.

Some properties of the lipid particles

The lipid particle suspension could not be fractionated by any of several mild methods. Electrophoresis at pH 9.0, 7.4 and 6.0 gave single ascending and descending boundaries which became less distinct with time and rapidly moved at different rates towards the anode, suggesting a polydisperse system bearing a negative charge. PBC remained in the fast-moving fraction. The pH value of maximum precipitation (iso-electric point?) was between pH 3 and 4, so that fractional precipitation at lower pH values would involve too much loss of PBC to be practicable. Differential centrifugation of lipid particles over the range 8000–16,000 *g* produced no changes in the PBC or lipid P assays. A fairly constant amount of lipid particle was soluble in each ml. of distilled water but the saturated solution was too dilute to produce a visible boundary in the electrophoresis apparatus. Both lipid particles and PBC were completely precipitated by calcium ions and by freezing and thawing, but were unaltered in appearance by boiling. A preparation of *Clostridium welchii* α -toxin (containing the lecithinase C) in 0.01 M-CaCl₂, or 1 mg. crystalline ribonuclease or trypsin per ml. at 37° for 30 min. had no effect on PBC or on the lipid particles.

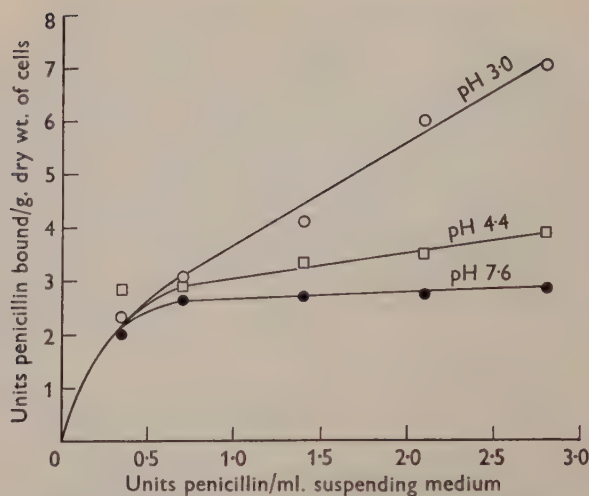


Fig. 3. Effect of pH value on the uptake of radiopenicillin on intact cells suspended at 5 mg. dry wt./ml. in 0.1 M-phosphate + 0.1 M-acetate. Three samples of cells were adjusted to pH values of 3.0, 4.4, 7.6 respectively, each sample was divided into five portions and differing concentrations of radiopenicillin was added to each portion. After 15 min. at 18° the cells were rapidly centrifuged and washed 3 times in water for ^{35}S assay.

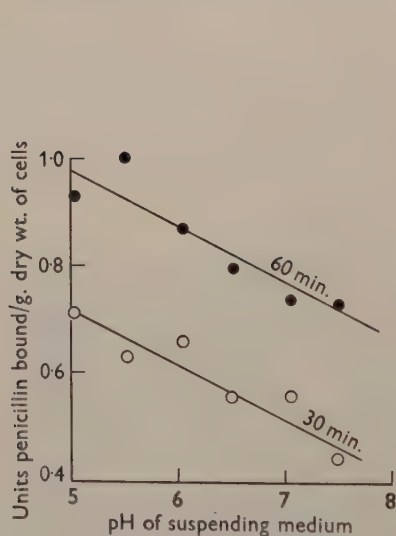


Fig. 4

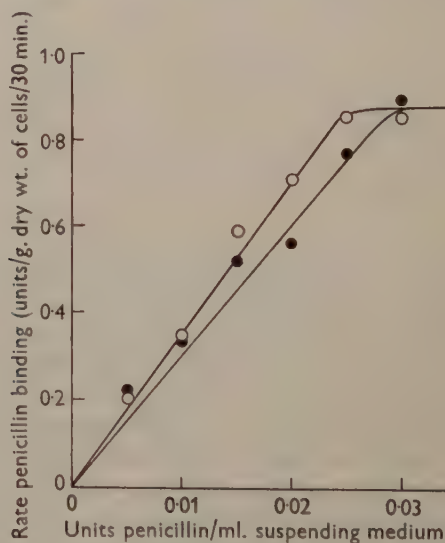


Fig. 5

Fig. 4. Effect of pH on rate of penicillin uptake. Six samples of cells were adjusted to various pH values in 0.1 M-phosphate + 0.1 M-acetate and radiopenicillin was added to 0.02 u./ml. at zero time. Half of each sample was rapidly centrifuged and washed after 30 min. at 18° (○—○), and the remainder after 60 min. (●—●), for ^{35}S assay.

Fig. 5. Effect of penicillin concentration on rate of uptake at pH 5.53 (○—○) and pH 7.35 (●—●). Cells were suspended in 0.1 M-phosphate + 0.1 M-acetate, the suspensions were adjusted to pH 5.53 or pH 7.35 and added to the prepared radiopenicillin dilutions. After 30 min. at 18° the samples were centrifuged rapidly and washed for ^{35}S assay.

Attempts to extract PBC from intact cells

At room temperature addition of ethanol, 5% ethanol, ethyl ether, chloroform, acetone, 5% phosphate (pH 9.0), 9% urea or 10% NaCl solutions to freeze-dried cells did not remove PBC; nor did adjustment of a thick cell suspension to pH 9, 10, 11 or 12 followed by neutralization of the separated extracts. Under these conditions, the organic solvents mentioned were also ineffective in removing lipid. Trichloroacetic acid (10%, w/v), anhydrous formic acid, anhydrous pyridine, *cyclohexylamine*, 90% phenol, hot methanol and anhydrous diethylene glycol rendered the cells incapable of irreversibly binding radiopenicillin, but no PBC (by bioassay; or by electro dialysis assay method, Few, Cooper & Rowley, 1953) was detected in the residues from extracts made with trichloroacetic or formic acids, *cyclohexylamine*, pyridine or 90% phenol (dialysed or freeze-dried to remove solvent), with diethylene glycol (dialysed or ethanol-precipitated) or with hot methanol (distilled off *in vacuo*).

Treatment with 90% phenol, followed by cold acetone and ether, did not extract any ^{35}S from cells grown in radiopenicillin and washed thoroughly, although all the lipid and lipid P was extracted. This suggests that the penicillin did not react with the lipid fraction of the lipid particles, although any solvent procedures which succeeded in extracting cell lipid (90% phenol, pyridine, hot methanol, *cyclohexylamine*) also succeeded in destroying PBC.

Uptake of penicillin by yeast

The findings of Maass & Johnson (1949a) that penicillin was not bound by yeast have been confirmed in this laboratory with a strain of baker's yeast. Daniel & Johnson (1954) showed that cell-free extracts of *Saccharomyces carlsbergensis* bound penicillin in an apparently specific manner, but radiopenicillin uptake experiments in the present work did not reveal any irreversible binding of penicillin by the lipid particles of the baker's yeast used (i.e. less than 0.1 unit penicillin at 0.4 unit/ml.). Perhaps yeast species contain PBC within the cell and sheltered from penicillin, rather than at or near the surface as in *Staphylococcus aureus*. It is interesting that briefly heating the supernatant from centrifuged ruptured staphylococcal cell suspensions appears to destroy the 'reversible' binding by cell contents (Daniel & Johnson, 1954; Cooper, 1954).

DISCUSSION

It can be seen that considerable difficulties are involved in the separation of PBC in a chemically definable form. All attempts to prepare a soluble extract were unsuccessful except for a simple saturated solution of lipid particles which was rather dilute to handle chemically or electrophoretically. Attempts to concentrate this solution produced coagulation on freeze-drying or inactivation by heat. PBC is very unstable to heat and acid, and cannot be kept long even at low temperature and at the optimum pH. Accordingly, extraction procedures must not be used which require prolonged standing in the presence of water, e.g. dialysis or solvent precipitation.

The so-called 'lipid particles' contain a large amount of protein (Mitchell & Moyle, 1951) as well as about 20% of lipid material and PBC, and their behaviour as outlined above indicates their chemical constitution to include a sparingly soluble lipo-protein complex in which the fatty material is strongly bound. PBC also appears to be strongly bound, but it may be more closely connected with the protein part. This is perhaps to be expected if penicillin specifically interferes with some enzymic activity, although binding is not affected by trypsin. Daniel & Johnson (1954) also found that their PBC was not affected by trypsin. The effect of trypsin may thus be a useful method of distinguishing the specific binding by PBC from the non-specific binding present in supernatants from ruptured cell suspensions (Cooper, 1954).

I should like to thank Dr D. Rowley for his helpful criticism, advice and support. I wish to acknowledge a grant from London University Central Research Fund for apparatus.

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A Serological Classification of Lactobacilli

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SUMMARY: A collection of 442 strains of lactobacilli, including representatives of all recognized species and 190 freshly isolated strains was studied serologically. Using sera prepared against some of these strains and crude HCl extracts, it was possible to classify 312 (70 %) of these strains by precipitin tests into six groups and one subgroup. This classification was in agreement with one based on the physiological characteristics of the strains.

When the serological identification of lactobacilli has been studied in the past, workers have concentrated mainly on limited aspects of the problem rather than on a broad study of the genus, and no comprehensive serological classification has yet been achieved. Results of earlier work in the different fields show conflicting or inconclusive results and the antigenic relations among lactobacilli cannot be deduced from them. More recently Williams (1948*a, b*), using agglutinin-absorption tests, investigated antigenic components in oral lactobacilli, and found four major antigens which he designated alphabetically (A, B, C, D), and by monospecific absorbed sera was able to give antigenic analyses for 18 of his 30 strains. Orland (1950) extended the antigenic analyses and was able to detect major antigens in 33 of the 252 strains studied; 30 of these, many of them designated as different species, possessed a common antigen F. Other major antigens were also distinguished, making, with those of Williams, a total of nine major antigens which could be identified in lactobacilli. Although minor antigens were observed in some strains, they could not be demonstrated consistently and the rest of Orland's strains remained unidentified.

Agglutination tests, and occasionally complement-fixation tests, have been used for serological studies of lactobacilli. The precipitin test has been used only infrequently, chiefly by Harrison and his co-workers (Harrison, Zidek & Hemmens, 1939; Harrison & Opal, 1944), who used this method to classify oral and intestinal strains of lactobacilli. They extracted a carbohydrate from the lactobacilli by acid extraction, purified it by reprecipitation with ethanol and used a solution of the residue as antigen against their precipitating sera. By quantitative precipitin tests they divided their strains into four serological types and a remaining heterogeneous group of unclassified strains. Niven, Castellani & Allanson (1949) used ring precipitin tests to identify strains of lactobacilli isolated from spoiled sausage meat. Although HCl extracts of all their 20 strains reacted with sera prepared against 2 of these strains, they concluded that this was a type reaction.

The purpose of the present work was to investigate serologically strains of lactobacilli from a wide variety of sources, comprising named strains of all

available species and freshly isolated strains of different origin. Preliminary studies of the antigenic reactions of lactobacilli included both agglutination and precipitin tests. Agglutination tests suggested a large number of heterologous organisms, and were considered to indicate type relationships; precipitin tests, however, indicated broader groups, usually compatible with physiological characteristics, and this method was used for further work.

METHODS

Organisms. The 442 strains of lactobacilli used for this work were described by Briggs (1953*a*) and included representatives of all available recognized species. They consisted of a collection of named strains maintained at this Institute, named strains obtained from the laboratory collections of other workers, and freshly isolated strains from different sources. Briggs (1953*a*) found that 390 out of 452 strains could be separated by physiological tests into eight groups, and the remaining sixty-two organisms into four sections. As these physiological groups of Briggs will be constantly referred to in the text, Table 1 summarizes the biochemical characteristics by which she defined her groups. In the sequel, in designating cultures, NCTC refers to the National Collection of Type Cultures, Colindale, London; NCIB to the National Collection of Industrial Bacteria, Teddington; ATCC to the American Type Culture Collection, Washington, D.C. and NIRD to the collection at the National Institute for Research in Dairying.

Table 1. *Physiological reactions characterizing the groups and sections defined by Briggs (1953*a*)*

Physio- logical group	Gas from glucose	NH ₃ from arginine	Survival at		Growth at			Tolerance of 4 % NaCl	No. of strains in group
			60° for 90 min.	65° for 30 min.	15°	45°	48°		
I	—	—	—	—	—	+	—	—	34
II	—	—	—	—	—	—	—	—	16
III	—	—	+	—	—	+	—	—	20
IV	—	—	—	—	+	+	+	+	25
V	—	—	+	+	—	+	+	—	82
VI	—	—	—	—	+	—	—	±	127
VII	+	+	—	—	+	—	—	±	55
VIII	+	+	—	—	—	+	—	±	31
Total number of strains grouped									390
Unclassified organisms									
Section 1	—	—	±	—	—	+	+	—	24
2	—	—	—	—	—	+	—	+	11
3	—	—	—	—	+	+	—	+	12
4	Miscellaneous reactions								15
Total number of strains classified in sections									62
— = negative; + = positive. (After Briggs, 1953 a.)									

— = negative; + = positive. (After Briggs, 1953*a*.)

Culture media. At first cultures to be used for immunization and for preparing HCl extracts were grown in a tomato Yeastrel broth containing Tween 80 (Briggs, 1953*b*). However, rabbits inoculated with thoroughly washed suspensions of lactobacilli grown in this medium produced antibodies against

tomato juice and Yeastrel, in addition to specific lactobacillus antibodies. These sera reacted with extracts of all lactobacilli, and with uninoculated media. To eliminate these non-specific reactions, which masked the specific lactobacillus precipitin reactions, cultures to be inoculated into rabbits were grown in medium A (below) which contained Yeastrel but no tomato juice, and cultures to be used to prepare HCl extracts in medium B which contained tomato juice but no Yeastrel. Antibodies to Yeastrel could thus be disregarded as no Yeastrel antigen was present in the extracts. Both media gave an adequate growth of all strains of lactobacilli.

Medium A (% w/v): Yeastrel, 0.3; glucose, 2.0; NaCl, 0.5; Tween 80, 0.01; Evans peptone, 1.5; final pH value 6.4–6.6. Evans peptone (Evans Medical Supplies Ltd., Liverpool 19) was found to be the most satisfactory for serological work with lactobacilli.

Medium B. This was the same as medium A except that 10.0 % (v/v) tomato juice replaced the Yeastrel.

Incubation temperatures. Strains of named lactobacilli were incubated at the following temperatures: *Lactobacillus acidophilus*, *L. bulgaricus*, *L. delbrueckii*, *L. fermenti*, *L. helveticus*, *L. lactis*, *L. leichmannii*, *L. odontolyticus* at 37°; *L. casei*, *L. plantarum*, *L. brevis* and *L. pastorianus* at 30°. Freshly isolated strains were incubated at temperatures appropriate to their sources until identified.

Preparation of group sera

Cultures of lactobacilli grown for 18 hr. in 40 ml. of medium A were centrifuged, washed twice in 0.85 % saline and suspended in 5–10 ml. saline. Suspensions were heated at 60° for 1 hr., stored at 4° and used for one course of injections. Suspensions were diluted to an equivalent of Brown's opacity tube no. 3 (Burroughs Wellcome and Co. Ltd.) and rabbits were given a course of five intravenous inoculations at three- to four-day intervals. If a test bleeding then indicated a satisfactory group serum, the rabbit was bled. If not, a fresh suspension of cells was prepared and one or more further courses of inoculation given. Sometimes group antibodies appeared after one course of inoculations, at others two or three courses were necessary, varying with the group and the strain used.

Group sera were tested for potency and specificity, and then stored at 4° with thiomersalate (1 in 10,000) as a preservative; potency was lost on storage, particularly with weak sera. Recently all freshly prepared sera have been freeze-dried within a few weeks of collection, and the results indicate that on reconstitution no loss of potency of group antibodies has occurred. In the earlier part of this work test bleedings were done on all rabbits before inoculation, and the sera tested against extracts of several different strains of lactobacilli for the presence of naturally occurring antibodies. As the results were always negative this practice was discontinued.

As group sera were not always easy to prepare some of the difficulties encountered are mentioned here. Inoculation of some strains of lactobacilli into rabbits gave antisera which reacted only with the homologous extract and sometimes with a few other extracts. Such sera, which were readily produced

after only a few inoculations, and which gave massive precipitin rings, were therefore regarded as type sera. After further courses of inoculation with some strains group antibodies appeared but with other strains none was detected even after several courses of inoculation; eventually non-specific antibodies common to many unrelated strains of lactobacilli were produced. Some strains, such as those from the NCTC or ATCC, which had been cultivated *in vitro* for some years and appeared to have lost most or all of their type antigens, gave better group sera than freshly isolated strains which usually gave potent type sera. Strains which produced only type sera reacted, however, with their specific group sera, indicating that group antigen was present.

In some instances the incubation temperature of cultures for inoculation was important, one temperature favouring the formation of group antigen and another the formation of type antigen. *Lactobacillus helveticus* cultures grown at 37° stimulated the production of a predominantly type serum; with organisms grown at 30° a potent group serum with less type activity resulted. With *L. casei*, on the other hand, incubation at 37° gave cells which favoured the formation of group antibodies in the serum, whereas incubation at 30° gave cells which induced the appearance of type antibodies.

Sometimes the number of inoculations necessary to produce group sera yielded sera which also cross-reacted with extracts of strains from other groups. The fact that such antibodies took longer to appear in the sera, and gave weak and irregular reactions with extracts of different strains in a group, suggested that they were due to minor non-specific antigens, which Williams (1948*a*) and Orland (1950) noted. A knowledge of the behaviour of individual strains and careful control of antibody production by frequent test bleedings usually overcame this trouble, although such cross-reactions could be removed from sera by absorption with suitable strains. Unabsorbed grouping sera were used whenever possible, because it was found on several occasions that absorption with suspensions of heterologous strains greatly weakened the group antibody of a serum even though it gave no precipitin reaction with extracts of the absorbing strain.

It was sometimes found, when test bleedings had indicated that only a weak group antibody had been produced and another course of inoculations was necessary, that after another course of inoculations the group antibodies disappeared leaving only type antibodies, and further injections would not bring back the group reaction. A similar observation was made by Jacob (1947) whilst preparing sera against group E streptococci.

Absorption of sera. Cells for absorbing sera were grown for 48 hr. in medium B (chosen because it gave a more profuse growth than medium A), washed twice in saline and the packed cells added to serum in the ratio of 1 vol. cells:4 vol. serum. Sera were absorbed for 2 hr. at 37° followed by overnight refrigeration; if necessary they were reabsorbed with fresh cells.

Preparation of extracts

Several different methods of extracting the group antigens from the cells were tried: formamide (Fuller, 1938); trichloroacetic acid (van den Ende, 1952); disintegration of the cells on a tissue disintegrator (Mickle, 1948; cf.

Shattock, 1949); Lancefield's (1933) HCl extraction method. The last was the most satisfactory and was used throughout this work. Cells for extraction were grown for 48 hr. in 40 ml. medium B, centrifuged and extracted with 1.0-2.0 ml. 0.05 N-HCl in saline in a boiling water-bath. Extracts were neutralized, thiomersalate (to 1 in 10,000) added and stored at 4° in stoppered tubes.

Ethanol precipitation of crude HCl extracts. As described by Harrison *et al.* (1939) a few of the crude HCl extracts were purified by precipitating with 4 vol. ethanol, the precipitate was then dissolved in saline, and any undissolved precipitate removed by centrifuging (fraction A); the ethanol supernatant was evaporated to dryness and redissolved in saline (fraction B).

Precipitin tests

Extracts were layered on serum in small glass tubes of 3.0 mm. internal diameter and kept at room temperature. The junction was examined for ring formation at intervals up to 30 min.

RESULTS

On testing crude HCl extracts of lactobacilli against specific grouping sera by the precipitin test it was found possible to classify 312 of the 442 strains examined into six groups and one subgroup. The following serological groups, named after the species from which the grouping sera were prepared were found: bulgaricus, casei-helveticus, casei, fermenti, plantarum and lactis-brevis, subgroup brevis. Table 2 shows the number of strains in each group.

Table 2. *Serological grouping of strains of lactobacilli by precipitin tests, using specific group sera*

Precipitin reactions of HCl extracts against specific sera of each serological group of lactobacilli

Number of strains reacting with sera							lactis-brevis	
	bulgaricus	casei-helveticus	casei	fermenti	plantarum		Unabsorbed serum	Absorbed serum
26	+	—	—	—	—		—	—
45	—	+	—	—	—		—	—
14	—	—	+	—	—		—	—
22	—	—	—	+	—		—	—
79	—	—	—	—	+		—	—
83	—	—	—	—	—		+	+
43	—	—	—	—	—		+	—
312 Total								

LACTOBACILLUS BULGARICUS GROUP

Group sera were prepared from *Lactobacillus bulgaricus* NCTC 2889 (ATCC 531) and from *L. bulgaricus* strain Icbna (received from Prof. Sherman). Reciprocal absorption tests showed that these two organisms possessed the same group antigen but a different type antigen.

Twenty-six strains of lactobacilli fell into this group, HCl extracts of all of them reacting specifically with *Lactobacillus bulgaricus* group sera. These 26 organisms consisted of 13 strains named *L. bulgaricus*, 2 of *L. helveticus*, 2 of *L. acidophilus*, and 9 freshly isolated strains from milk, soured milk, présure, and intestine of a pig fed with milk soured with a lactobacillus. According to the physiological tests of Briggs (1953*a*) 5 of these 26 strains fell into her group I, 8 into group II, 9 into group III, 2 into group V, 1 into group VI and 1 into section 4. Most of them (22 out of 26) are therefore included in groups I, II and III, which, as Briggs pointed out, are not as clearly distinguished from each other as some of her other groups. Since then Wheeler (1955), working on the same collection of strains, has differentiated *L. acidophilus* from *L. bulgaricus* by carbohydrate fermentations, salt tolerance and resistance to bile; 23 of the 26 strains in the present serological group *L. bulgaricus* were included in Wheeler's species *L. bulgaricus*.

LACTOBACILLUS CASEI-HELVETICUS GROUP

Extracts of 45 strains of lactobacilli gave a specific reaction with a serum prepared with *Lactobacillus casei* NIRD R 094 as antigen, and also with a serum prepared against *L. helveticus* ATCC 7469 (NCTC 6375). Reciprocal absorption tests (Table 3) showed that these two strains possessed the same group antigen but that each had its own type antigen. Absorption of the group sera with cells of either NIRD R 094 or of ATCC 7469 removed the group but not the type antibodies. As specific sera for this group were prepared from strains named both *L. casei* and *L. helveticus*, the serological group was named *L. casei-helveticus*. Absorption tests with cells of *L. casei* H831, one of the strains used to prepare serum for the *L. casei* group serum, were also included as controls, and showed that *L. casei* H831 had no antigenic relationship with the *L. casei-helveticus* group.

It was found that 28 of the strains falling into the *Lactobacillus casei-helveticus* group possessed the same type antigen as *L. helveticus* ATCC 7469. Table 3 shows that when a type-specific serum was prepared (by a very short course of inoculations) with this strain only, strains having antigens of that type reacted; absorption of the serum with cells of NIRD R 094 had no effect, whereas absorption with ATCC 7469 cells removed the type antibodies. The 16 strains which did not possess the ATCC 7469 type antigen were found to have at least 3 other type antigens.

The possession of the ATCC 7469 type antigen was mainly associated with the physiological characters defining Briggs group IV: 22 of the organisms belonging to this serological type fell into her group IV, only 3 into group VI, and the remaining 4 into sections 3 and 4. The strains having other type antigens, however, belonged mostly to group VI: 10 fell into this group and only 3 into group IV, whilst 3 were in sections 3 and 4. Although some overlapping occurs, the differentiation is clear enough to suggest that the *Lactobacillus casei-helveticus* group might be further divided to identify strains belonging to the serological type ATCC 7469 as *L. helveticus*, and strains belonging to other serological types as *L. casei-helveticus*. The ability of strains

of *L. helveticus* to grow at high temperatures has important practical applications and a serological differentiation correlated with such a characteristic would be greatly valued. The examination of further strains is necessary, however, before the wider validity of such a distinction could be established.

Strains in the *Lactobacillus casei-helveticus* serological group which did not possess the ATCC 7469 type antigen consisted of 6 named strains of *L. casei*, 2 of *L. helveticus*, 2 of *L. acidophilus*, 1 of *L. odontolyticus*, 2 oral strains and 3 other strains isolated from milk and cheese. Strains belonging to serological type ATCC 7469 consisted of 10 named strains of *L. helveticus*, 10 of *L. casei*, 2 of *L. acidophilus*, 1 of *L. bulgaricus*, 1 of *L. odontolyticus*, 2 of *L. delbrueckii*, 1 oral strain and 2 freshly isolated strains from yoghurt and Stilton cheese respectively. Nine of the organisms in this latter section (3 of *L. casei* and 6 of *L. helveticus*), although received from various sources, are believed (Briggs, 1953a) to be cultures of *L. casei* ATCC 7469 as all bore this collection number. It is of interest to note that they have all retained their type and group antigens under diverse conditions of maintenance.

Table 3. Reciprocal absorption tests showing the antigenic relationship between strains of *L. casei* and *L. helveticus*

Strains tested	Group serum <i>L. casei</i> NIRD R094				Group serum <i>L. helveticus</i> 7469				Type serum <i>L. helveticus</i> 7469			
	Absorbed with			Un- absorbed	Absorbed with			Un- absorbed	Absorbed with			Un- absorbed
	R094	7469	H831		R094	7469	H831		R094	7469	H831	
<i>L. casei</i>												
NIRD R094	+	-	+	+	+	-	-	+	-	-	-	-
15 other strains	+	-	-	+	+	-	-	+	-	-	-	-
<i>L. helveticus</i>												
ATCC 7469	+	-	-	+	+	+	-	+	+	+	-	+
28 other strains	+	-	-	+	+	+	-	+	+	+	-	+
<i>L. casei</i>												
H831	-	.	.	.	-	.	.	.	-	.	.	.

LACTOBACILLUS CASEI GROUP

The *Lactobacillus casei* serological group is quite distinct from the *L. casei-helveticus* group; no cross-reactions occurred between sera of the two groups and absorption with cells of strain H831 did not remove the group antibodies from *L. casei-helveticus* sera (Table 3). Extracts of 14 strains of lactobacilli reacted with two group sera prepared against *L. casei* NIRD H831 and *L. casei* NIRD DECP respectively. Organisms included in this group consisted of 6 strains received as *L. casei*, 1 as *L. lactis*, 2 as *L. plantarum* (both having the same NCTC number but from different sources), 1 oral strain, and 4 freshly isolated strains from fresh and soured milk. Of these, 12 fell into Briggs group VI and 2 into her section 3.

LACTOBACILLUS FERMENTI GROUP

Twenty-two strains of lactobacilli reacted specifically with a serum prepared against a strain of *Lactobacillus fermenti* (*L. fermentatus* ATCC 9338; NCTC

6991), and also with a serum prepared against a freshly isolated oral strain 010, which showed similar physiological characteristics. All the strains in this serological group were heterofermentative organisms, 21 being included in Briggs group VIII and 1 in Briggs group VII. However, 5 other strains identified by other workers as *L. fermenti*, and 3 freshly isolated strains also in Briggs group VIII, did not react with either of these sera. It was noted that 7 of these strains were of intestinal origin, whereas only 2 of the 22 reacting strains were intestinal. It is possible that there is another serological group consisting mainly of intestinal strains of *L. fermenti* as opposed to those of oral, milk and plant origin, although I have not yet succeeded in preparing a corresponding serum. Strains in the *L. fermenti* group consisted of 10 strains named *L. fermenti*, 2 of *L. gayonii*, 1 of *L. mannitopoeus*, 1 of *L. lycopersici*, 4 freshly isolated strains from Gruyère cheese, 2 oral strains and 1 strain each from pig faeces and chick duodenum.

Prolonged inoculation of rabbits with organisms in this group led to the production of antibodies which reacted weakly with extracts of a number of strains of the *Lactobacillus casei-helveticus* group. Similarly, weak antibodies which reacted with extracts of *L. fermenti* could be detected in *L. casei-helveticus* antisera after long courses of inoculation, indicating a minor antigen common to these two groups.

LACTOBACILLUS PLANTARUM GROUP

Into this group fell 79 strains of lactobacilli, all of which reacted specifically with a serum prepared against *Lactobacillus plantarum* NCTC 7220. Attempts to prepare similar group sera against other laboratory or freshly isolated strains of *L. plantarum*, extracts of which reacted strongly with this serum, proved unsuccessful. All strains in this serological group fell into Briggs group VI, and included 17 strains named *L. plantarum*, 4 of *L. arabinosus*, 2 of *L. pentosus*, 3 of *L. casei*, 3 of *L. odontolyticus*, 2 of *L. pastorianus* and 1 of *L. fermenti*. Freshly isolated strains were obtained from silage, raw and soured milk, and Cheddar cheese.

It was noted that when inoculation of rabbits with strain NCTC 7220 continued for longer than was necessary to obtain a *Lactobacillus plantarum* group antiserum other antibodies were detected in the serum. Only extracts of strains of *L. plantarum* and of organisms of similar physiological characteristics reacted with early bleedings, after a further course of inoculation extracts of strains of organisms of the *L. lactis-brevis* group (described below) also reacted. It was possible to remove this cross-reaction by absorption with a strain of *L. lactis* or *L. brevis* but the specific reaction was then also somewhat weakened, and it was found advisable to prepare group sera by using as short a course of inoculations as possible. It appeared that the *plantarum* strain possessed a minor antigen in common with *L. lactis* and *L. brevis*; this was confirmed by further work on these two organisms.

LACTOBACILLUS LACTIS-BREVIS GROUP

Using the *Lactobacillus lactis* strain Dorner type I (ATCC 8000, NCTC 7278) a group serum was prepared for the *L. lactis* serological group. Another strain,

L. lactis NIRD 244, stimulated similar but more potent antisera. Reciprocal absorption tests showed these two organisms to be identical. Eighty-three strains reacted specifically with these sera, including 18 of 19 strains received as *L. lactis*, 5 received as *L. bulgaricus*, 2 as *L. helveticus* and 1 as *L. acidophilus*. Freshly isolated strains were from calf abomasum, pr  sure, dried calf stomach, raw milk, Gruy  re cheese. Of these strains 70 fell into Briggs group V, 2 into group III, 8 into section 1, and 1 into section 4. Thus strains in this serological group were all homofermentative and mainly thermophilic organisms, unable to hydrolyse arginine, able to grow at 48   and to resist heating to 60   for 1   hr. (Table 1). It seemed that another serological group containing only organisms closely allied in physiological characteristics had

Table 4. Reciprocal absorption tests showing antigenic relationship between strains belonging to the *L. lactis* and *L. brevis* serological group

Strains tested	Sera											
	<i>L. lactis</i> 244				<i>L. buchneri</i> 4005				<i>L. plantarum</i> 7220			
	Absorbed with				Absorbed with				Absorbed with			
	Un- absorbed	lactis	<i>brevis</i>	planta- rum	Un- absorbed	lactis	<i>brevis</i>	planta- rum	Un- absorbed	lactis	<i>brevis</i>	planta- rum
12 of <i>L. lactis</i>	++	-	-	+	++	-	-	+	-	.	.	.
8 of <i>L. brevis</i>	++	-	-	-	++	-	-	-	-	.	.	.
5 of <i>L. plantarum</i>	-	.	.	.	-	.	.	.	++	+	+	-

been established. However, it was observed that these otherwise specific sera gave strong cross-reactions with another group of organisms which differed markedly from the *L. lactis* group in physiological reactions, being heterofermentative, hydrolysing arginine, growing at 15   but not at 45  , and unable to withstand heating to 60   for 30 min. In contrast with the rapid growth of the *L. lactis* organisms they grew very slowly. All these heterofermentative strains fell into Briggs group VII, and included named strains of *L. brevis*, *L. acidophil-aerogenes*, *L. bifidus*, *L. rudensis*, *L. buchneri*, *L. hilgardii*, *L. caucasicus*, *L. kefir*, *L. frigidus*, *L. malefermentans*, *L. parvus* and 13 strains of *L. pastorianus*. Freshly isolated strains were obtained from beer, silage, raw and soured milk, yoghurt and Cheddar cheese. It would scarcely be possible to find within the lactobacillus genus two groups more divergent biochemically, and yet they appeared to possess a common major antigen. It was not, as with the *L. plantarum* group, the question of a shared minor antigen giving rise to slight or delayed cross-reacting antibodies in the sera, but extracts of these heterofermentative strains in the *L. brevis* group reacted as strongly as extracts of the *L. lactis* group with the *L. lactis* sera, and at the same stage of preparation of the antisera. Sera were prepared against two of the heterofermentative organisms, *L. brevis* ATCC 367 (NCIB 8169) and *L. buchneri* ATCC 4005 (NCIB 8007). Both these sera also gave strong reactions with extracts of organisms of the *L. lactis* serological group, as well as with those of the *L. brevis* group. The reciprocal absorption tests (Table 4) between *L. lactis*

NIRD 244 and *L. buchneri* ATCC 4005, and their antisera showed complete cross-absorption. Various organisms were used in an endeavour to obtain differential absorption between sera against the two groups of organisms, and partial success was eventually obtained by absorbing with *L. plantarum* NCTC 7220. It was found that this strain absorbed antibodies which reacted with the extracts of the *L. brevis* strains, whereas the *L. lactis* extracts still reacted, although not so strongly. Table 4 shows that sera prepared against either *L. lactis* or *L. brevis*, after absorption with *L. plantarum* NCTC 7220 cells, reacted only with the *L. lactis* strains and not with the *L. brevis* strains. This absorption was not easy to accomplish: when the proportion of cells to serum was too small, extracts of the *L. brevis* strains also reacted with the serum; when too great, the precipitin reaction for the *L. lactis* strains was greatly weakened. Other strains of *L. plantarum* had a similar effect, but strain NCTC 7220 was found to give the best results. By means of this quantitative absorption it was possible to separate these two groups.

Table 5 gives a summary of the distribution of the physiological groups of Briggs among the serological groups of lactobacilli found in the present work.

UNCLASSIFIED STRAINS

Of the strains examined here 70% have been classified into serological groups. The remaining 130 organisms have not yet been identified serologically. Of these, 73 named strains included 17 strains of *Lactobacillus acidophilus*, 8 of *L. delbrueckii*, 5 of *L. bulgaricus*, 6 of *L. leichmannii*, 5 of *L. fermenti*, 5 of *L. helveticus*, 2 of *L. jugurt*, 7 of *L. casei*, 11 of *L. plantarum*, 2 of *L. pentosaceus*, and 1 each of *L. lactis*, *L. arabinosus*, *L. brevis*, *L. bifidus* and *L. lycopepersici*. Fifty-seven freshly isolated strains also remained unclassified, 34 from intestinal sources, and the remaining 23 from silage, milk, cheese, greening sausage, and sauerkraut. The distribution of these serologically unclassified strains among the Briggs physiological groups is shown in Table 5.

Table 5. *Association of physiological characteristics with the serological grouping in 442 strains of lactobacilli*

Serological group	Number of strains falling into each of the Briggs physiological groups													Not tested	Total
	Physiological group								Section						
	I	II	III	IV	V	VI	VII	VIII	1	2	3	4			
bulgaricus	5	8	9	.	2	1	1	.	26	
casei-helveticus	.	.	.	25	.	13	5	2	.	45	
casei	12	2	.	.	14	
plantarum	79	79	
lactis	.	.	2	.	70	.	.	.	8	.	.	1	2	83	
brevis	42	1	43	
fermenti	1	21	22	
Not grouped	28	8	9	.	8	22	12	8	12	9	4	6	4	130	

Reproducibility of results

Extracts of all strains of lactobacilli were again made at least a year after first testing, and retested against the same sera and against fresh sera prepared

with the same strains. With a few exceptions precipitin tests gave the same serological reactions as on first testing. Nine strains of the *Lactobacillus plantarum* serological group (including 7 freshly isolated ones), which had originally reacted strongly with the serum, reacted only weakly or not at all on retesting, and one strain of *L. casei-helveticus* group was found to have lost both type and group antigens. None of these strains had acquired the antigens of any other group. Freshly isolated strains tested on first isolation and again after laboratory cultivation were, with the exception of the strains quoted above, unchanged serologically as far as the group antigen was concerned.

It has recently been found that when one unidentified strain was used to prepare a group serum, this reacted with acid extracts of all strains within the *Lactobacillus casei* group, but not with the homologous extract. It was then found that by treating the cells of the homologous organism by other methods, such as autoclaving at 120° for 5 min. (Rosendal, 1950), grinding, or use of the tissue disintegrator, it reacted strongly with the homologous antiserum. The reason for this occasional failure of HCl extraction, which did not affect other strains of the same serological type, is not yet known.

Crossing antigen R

Mention has already been made of minor antigenic cross-reactions which occurred between organisms in different serological groups. In addition to these a major antigen (named R by the author) was noted in a number of organisms of different serological groups. Sera containing R antibodies reacted selectively with a number of strains belonging to different groups, giving a strong precipitin reaction. Although its distribution appeared to be random, extracts of all strains were tested for its presence, as further work may indicate some biochemical characteristic common to all these strains. Sera against this antigen were prepared by using a strain of an unidentified *Lactobacillus* sp., AH4, isolated from Gruyère cheese and falling into section 1 of Briggs, and a strain of *L. bulgaricus* SM10 isolated from soured milk. The 37 strains in which this antigen was demonstrated fell into the following serological groups: 8 in *L. bulgaricus*, 2 in *L. casei*, 9 in *L. lactis*, 3 in *L. fermenti* and 2 in *L. plantarum* group; the other 13 strains were unidentified serologically. Most of the identified strains were members of the *L. bulgaricus* and *L. lactis* serological groups, but strains from all other groups except *L. casei-helveticus* and *L. brevis* were represented. It is interesting that although the origin of some of these strains is not known, 20 of them were known to be isolated from milk or milk products, and only one was of intestinal origin.

Ethanol precipitation of crude extracts

On testing fractions A (ethanol insoluble) and B (ethanol soluble), prepared by Harrison's method (Harrison *et al.* 1939; see Methods) against group and type sera it was found that the group antigen was precipitated by ethanol; only fraction A reacted with the group sera. This agrees with similar findings by Shattock (1949) with group D streptococci that the group antigen was contained in the ethanol precipitated fraction. The type substance was present in

both fractions. This method of concentrating the group antigen was tried with several ungrouped strains, but in no case did it reveal the presence of group antigen not already indicated in the crude unconcentrated extracts.

Comparison of precipitin and agglutination tests

Early in this work the results of agglutination tests were compared with those of precipitin tests, and it was found that agglutination tests indicated much narrower relationships among the lactobacilli than the precipitin reactions, and probably indicated type antigens. Later, a few strains of each group were tested against their group sera by agglutination test and the results confirmed the findings of the earlier work. Strains belonging to the same serological group but having different type antigens reacted only weakly in agglutination tests, usually to a titre of less than 1/80, to potent precipitating group sera. It appears that, as with the streptococci (Lancefield, 1941), the group reactions are not indicated by agglutination reactions. It was also found that the type reactions were not entirely in agreement in the two tests. Even when homologous type sera were used, the presence of a high agglutinin titre in a serum was not always associated with a strong precipitin reaction, and several type sera which gave rapid and massive precipitin reactions were only of low agglutinin titre. This suggests the presence of more than one type antigen, the presence of which is indicated only by the appropriate test, analogous with the M and T antigens of the group A streptococci.

Comparison with results of other workers

Few comparisons could be made with the serological results of other workers, since so little extensive serological work has been done with lactobacilli. However, Dr Orland kindly supplied me with 7 of the strains identified by Orland (1950) or by Williams (1948*a*) as possessing some of the antigenic components they mentioned. Table 6 shows a comparison of my serological grouping with their findings; 4 of Orland's strains fall into the *Lactobacillus casei-helveticus* group (2 of them being *L. helveticus*), 2 are *L. plantarum* and 1 is *L. casei*. As might be expected, there is little agreement between my precipitin and Orland's agglutination tests, which do not indicate the group relations between his organisms. It is of interest that the two *L. helveticus* strains (of which L320 bears the number ATCC 7469) were the strains with the F antigen, and it seems very probable that this F antigen may correspond to the *L. helveticus* type antigen. Orland mentioned that of the 30 strains which possessed this antigen, 9 were labelled ATCC 7469 and were presumably cultures of the same organism from different laboratories. In this work it has already been mentioned that 9 of the strains received here as either *L. casei* or *L. helveticus* were believed to be strains of this organism, and all of these were identified as *L. helveticus*. The other antigens of Orland and of Williams shown by agglutination tests are presumably type antigens, and are not indicated in the precipitin tests, i.e. 45A is not related to 3A1SB although both possess Orland's B antigen.

Table 6. *A comparison of the serological classifications of certain lactobacilli by Orland and by Williams and in the present work*

Strain	Source of identification	Serological classification	
		Orland and Williams antigen	Serological group of present work
3A1SB	Oral	AB	casei-helveticus
45A	Oral	BD	casei
EH22G	Oral	E	plantarum
L320 ATCC 7469	<i>L. casei</i>	F	casei-helveticus H*
L304	<i>L. plantarum</i>	G	plantarum
L323	<i>L. casei</i>	H	casei-helveticus
L641	Oral	FI	casei-helveticus H*

*H=possessing the helveticus type antigen.

Serological identity of differing colonial forms

It is of interest that my colleague Dr D. M. Wheeler plated out all the 442 strains examined here and found at least two widely different colonial forms in a number of strains. When the different variants of 25 strains were examined serologically, it was found in each case that the group antigen was the same for all the colonial variations in a strain; 1 strain exhibited four different colony forms, but extracts of each reacted with the same group serum.

DISCUSSION

Most of the difficulties mentioned in the preparation of the lactobacillus grouping sera are generally known to occur in the preparation of immune sera, particularly with the streptococci: Briggs & Newland (1952) mentioned the presence of antibodies which reacted with extracts of groups L and K streptococci in group N sera prepared against *Streptococcus cremoris*. With the lactobacilli the ease with which such minor cross-reacting antibodies develop, and the greater difficulty in preparing group sera with freshly isolated strains may have prevented this method of classification being used in the past. Once such specific group sera have been prepared the rapid identification of large numbers of lactobacilli presents no difficulty, the preparation of the crude extracts being quick and simple, and the troubles of the agglutination test, caused by unstable suspensions and autoagglutination, are avoided. The fact that the group antigens were present in old laboratory strains, which were as readily grouped as freshly isolated strains, showed them to be stable antigens and therefore suitable for serological identification. In this connexion it was noted that when several strains bearing the same code number or name were obtained from different laboratories, all such replicate cultures gave identical serological reactions; different methods of maintenance had not affected their specific reactions.

A similar division into broad groups forms the basis of successful serological classification of another member of the Lactobacteriaceae, the streptococci (Lancefield, 1933), and in view of the many characteristics shared by the two genera, it is not surprising that their serological attributes should be similar.

It is clear that lactobacilli possess a complexity of antigens, as Canby & Bernier (1942) concluded. In addition to the type and group antigens certain strains also have the R antigen, minor cross-reactions occur between groups, and there is evidence that other cross-reactions may occur selectively and yet unpredictably, cutting across the groups. It has been the practice to avoid preparing group sera from strains which contain such antigens, and to concentrate on the classification into broad groups.

The close agreement attained between the serological grouping and the physiological tests of Briggs is indicated in Table 5. That the two methods of classification were carried out concurrently and independently emphasizes the agreement between the serological and physiological findings. Each serological group is almost entirely represented in one physiological group, except that the *Lactobacillus bulgaricus* serological group organisms are distributed over the Briggs physiological groups I, II and III, and that strains in the *L. casei-helveticus* serological group occur in physiological groups IV and VI. It has also been possible to divide the large physiological group VI into three serological groups, *L. casei-helveticus*, *L. casei* and *L. plantarum*. Briggs (1953a) herself considered that physiological groups I, II and III, though plainly separated from the remaining groups, were not so clearly distinct from each other and that further confirmatory tests were necessary to distinguish them; Wheater (1955), in her work on the physiological characteristics of *L. acidophilus* and *L. bulgaricus*, has shown that the distribution of strains within the Briggs groups I-III is not the same as the distribution within the two species, so that strains of all three groups occurred in both *L. acidophilus* and *L. bulgaricus*. A close agreement was found between Wheater's defined species *L. bulgaricus* and the present serological group *L. bulgaricus*. In the case of *L. casei-helveticus* serological group, with strains distributed into two of the Briggs groups, it has been shown that it is possible to subdivide this serological group into *L. casei-helveticus* (containing mostly group VI strains) and *L. helveticus* (containing mostly group IV strains).

Thirty per cent of the strains tested still remain unclassified serologically. It will be noted that no mention has been made of a serological group corresponding to *Lactobacillus acidophilus*, and in fact 17 of the 22 strains received as *L. acidophilus*, and 23 homofermentative freshly isolated strains of intestinal origin, also probably *L. acidophilus*, remain as yet unidentified serologically. It has not yet been possible to prepare an antiserum which reacts with these strains. In some instances sera have been obtained which reacted specifically with some extracts, but in each case the number reacting was small, and may have been due to type reactions. Two such groups were defined among the intestinal lactobacilli, each containing 6 strains, but it was considered that neither group was large enough to constitute a named serological group. It may be that intestinal lactobacilli present peculiar problems in their serological grouping; it has been mentioned that several intestinal strains of *L. fermenti* could not be classified. The grouping of these organisms needs further work to determine their position.

None of the 6 strains of *Lactobacillus leichmannii* has been grouped, attempts

to produce a serum against one of them having been unsuccessful, and no group for *L. delbrueckii* has been defined. These may constitute further serological groups. Some unidentified strains may also have lost their group antigen; such loss of antigenicity has been experienced, particularly with strains of *L. plantarum*, although group antigen is normally very stable.

The close serological relationship of the biochemically divergent groups *Lactobacillus lactis* and *L. brevis* is of interest. Davis (1936) suggested that ecologically there were two broad groups of lactic acid bacteria, plant strains and animal strains, and that the use by man of milk, fermented mashes, and alcoholic drinks were artificial procedures which might select new species originating, however, from one of the two broad groups. Such a theory might account for a common origin of *L. brevis* and *L. lactis*, the two groups later becoming adapted to very different conditions. On the other hand, the sharing of antigenic components among different species or even genera of bacteria is not uncommon, e.g. the polysaccharide shared by pneumococcus type II and *Leuconostoc mesenteroides* (Sugg & Hehre, 1942), the polysaccharide common to pneumococcus type VI and *Haemophilus influenzae* type A (Chapman & Osborne, 1942), and a common type antigen shared between group D and group N streptococci (Sharpe, 1952). It seems however that, in view of this close relationship between homofermentative and heterofermentative groups of lactobacilli, it would be unwise to separate the homofermentative from the heterofermentative species, placing the latter in a separate genus as suggested by Rogosa, Wiseman, Mitchell, Disraely & Beaman (1953).

Harrison's (Harrison *et al.* 1939; Harrison & Opal, 1944) work on oral and intestinal strains of lactobacilli, with the use of purified HCl extracts, apparently indicated type and not group antigens. This was probably due to several causes: (i) as he used quantitative precipitin tests the group antigen, being much weaker than the type, would be diluted out more readily and the group reaction, when present, would only occur in the higher concentrations of antigen and be considered of minor importance; (ii) cultures used for preparing sera had been isolated within the previous 12 months and would have tended to stimulate the production of type rather than group antibodies; (iii) the inoculating suspensions were heated to 65° for 1 hr., and this has been found, at least in some cases, partially to destroy the group antigen.

The definition of these serological groups, substantiated by physiological tests and compatible with sources of origin, should remove much of the confusion existing in the serological relationships of the lactobacilli and serve as a basis for further elucidating their complex antigenic patterns.

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The Characteristics of *Lactobacillus acidophilus* and *Lactobacillus bulgaricus*

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SUMMARY: A study of 94 strains of lactobacilli was made. These were divided into three groups by 'sugar' fermentations and tolerance of sodium chloride and sodium tauroglycocholate. Strains of *Lactobacillus acidophilus* fermented amygdalin, cellobiose, salicin and sucrose, were variable in their action on dextrin and maltose, but grew in 2 % sodium chloride or sodium tauroglycocholate. Strains of *L. bulgaricus* fermented none of these sugars and failed to grow in 2 % sodium chloride or in 2 % sodium tauroglycocholate. A third intermediate group failed to ferment amygdalin, cellobiose, salicin or sucrose, but all fermented dextrin and maltose and grew in 2 % sodium chloride. They failed to grow in 2 % sodium tauroglycocholate.

From the early days of bacteriology, there has been difficulty in differentiating between *Lactobacillus acidophilus* and *L. bulgaricus* and there has, therefore, been some confusion between these organisms. In 1910 Metchnikoff suggested that *L. bulgaricus* could become established in the gut, but other findings, reviewed by Rettger & Cheplin (1921), suggested that only *L. acidophilus* could be implanted. The controversy which then arose was probably due to the lack of tests which would identify these organisms.

Several authors have suggested methods of differentiating between *Lactobacillus acidophilus* and *L. bulgaricus*, and some of these rely on a single characteristic. Sugar fermentations were used by some workers, including Rahe (1918) who found fermentation of lactose, sucrose and raffinose variable. He therefore suggested that they should be identified on their ability to ferment maltose. Kulp & Rettger (1924) studied a number of strains of these organisms and concluded that the reactions of the two species were very similar, the differences being in degree rather than in kind. They found that maltose, sucrose and laevulose were fermented by *L. acidophilus*, but rarely by *L. bulgaricus*. This was confirmed later by Sherman & Hodge (1940). Other workers who used sugar fermentations included Curran, Rogers & Whittier (1933), but their results were very different from those of Kulp & Rettger (1924). These conflicting results threw doubt on the value of these tests for identification, although it is probable that the variation was due to the inadequacies of, and differences in, the media used rather than to the fermentative variability of the organisms. Orla-Jensen (1919), for instance, found difficulty in reaching any conclusions in the fermentation tests he made, because his medium failed to support good growth. A further probable reason for some discrepancies is the attempt to fit the results to the names previously assigned to the organisms in some earlier work.

Sherman & Hodge (1940) found that *Lactobacillus acidophilus* would initiate

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growth at pH 7.5, whereas *L. bulgaricus* would not, but 'weak' strains of *L. acidophilus* behaved like *L. bulgaricus*. They also found growth temperatures to be unreliable. Tests for growth in 2.5% sodium chloride, for the quantity of carbon dioxide produced, and for ability to grow in a simple medium, gave more precise results.

The most recent study of *Lactobacillus acidophilus* was that of Rogosa, Wiseman, Mitchell, Disraely & Beaman (1953), who described its characteristics and on the basis of fermentations of single sugars recognized four variant types. Their work, however, was confined to oral strains and was not concerned with *L. bulgaricus*.

Briggs (1953*b*) was one of the few who attempted to classify the genus *Lactobacillus* as a whole; she divided these organisms into eight main groups by physiological tests (Table 1). Most strains labelled *Lactobacillus acidophilus* and *L. bulgaricus* came into either group I, II or III, and these groups were divided from the remaining homofermentative strains either by their inability to grow at 15° or 48°, or in 4% sodium chloride, or by their low resistance to heat. The three groups were separated from each other mainly by their maximum growth temperatures, and both groups I and II contained named strains of *L. acidophilus* and *L. bulgaricus*, whilst group III contained a few strains of the latter. Amongst the unclassified organisms in section I there were further strains of *L. bulgaricus*. It was concluded that further tests were required to confirm these groupings.

The descriptions of *Lactobacillus acidophilus* and *L. bulgaricus* given in *Bergey's Manual* (Breid, Murray & Hitchens, 1948) do not embody details which permit the clear identification of the organisms. The sugars used give variable results, and the 'distinctive characters' do not admit of easy routine differentiation. The aim of the present work was to define *L. acidophilus* and *L. bulgaricus* more clearly and to add, by way of simple tests, to the few known differential characteristics.

METHODS

Cultures and maintenance. The 94 cultures studied in this work were those placed in groups I–III and section 1 (Table 1) by Briggs (1953*b*). These groups consisted of a number of strains received as *Lactobacillus acidophilus* or *L. bulgaricus*, a few of *L. delbrueckii*, *L. helveticus*, *L. lactis* and *L. jugurt* and other unnamed and freshly isolated strains. For comparison, a few further cultures of named strains of lactobacilli were examined by microtests.

The details of the maintenance procedure and the media used for the routine culture and storage of these organisms were given by Briggs (1953*b*). The media used in the tests reported here are given under the test headings. The inoculum for all tests, except the microtests, was 0.05 ml. of a vigorously growing culture, usually after incubation for 18 hr. at 37 or 45°, or after 42 hr. at 30°. All tests were repeated at a later stage to confirm that the results were reproducible.

In all these tests a good medium for growth is essential. It is also imperative that the culture used for the inoculum is growing well. This was demonstrated

clearly at one stage in this work: two cultures, which were difficult to classify but were thought to be *Lactobacillus acidophilus* or *L. bulgaricus*, gave fermentation results very different from other strains of these species. When the cultures were re-tested by the methods of Briggs (1953*b*), but with heavier inocula of actively growing cultures, they were found to be strains of the heterofermentative species *L. fermenti*. It has also been found that tomato juice used in the basal medium gives much better growth after a period of storage at 4° instead of at room temperature.

Table 1. *Physiological classification of the lactobacilli**

	Gas from glucose	NH ₃ from arginine	Survival at		Growth at			Tolerance 4% NaCl	Number of strains
			60° for 30 min.	65° for 30 min.	15°	45°	48°		
Group I	—	—	—	—	—	+	—	—	34
Group II	—	—	—	—	—	—	—	—	16
Group III	—	—	+	—	—	+	—	—	20
Group IV	—	—	—	—	+	+	+	+	25
Group V	—	—	+	+	—	+	+	—	82
Group VI	—	—	—	—	+	—	—	±	127
Group VII	+	+	—	—	+	—	—	±	55
Group VIII	+	+	—	—	—	+	—	±	31
Section 1	—	—	±	—	—	+	+	—	24
Section 2	—	—	±	—	—	+	—	+	11
Section 3	—	—	—	—	+	+	—	+	12
Section 4			Miscellaneous reactions						15

* After Briggs (1953*b*).

Microtests. These included the methyl red test, hydrolysis of starch, utilization of citrate, production of indole, production of acetoin, reduction of nitrate and production of H₂S. The tests were carried out according to Clarke & Cowan (1952) except that the cell suspensions were prepared as follows. Plates of tomato glucose agar were inoculated by spreading 0.5 ml. of 18 hr. cultures over the surface. The plates were incubated anaerobically; those cultures which grew at 37 and 45° were incubated for 24 hr. and those which grew at 30° for 48 hr. Growth was washed from the surface of the plates with sterile distilled water and the suspension centrifuged. The cells were then resuspended in distilled water, diluted to a standard opacity on an absorptiometer, recentrifuged and finally resuspended in one-tenth the volume of distilled water necessary to give the standard opacity. This concentrated suspension was used for the microtests.

Carbohydrate fermentation. The basic medium, consisting of Neopeptone 1.5%, Tween 80 0.1%, Yeastrel 0.6%, and agar 0.15%, had a final pH of 7.0–7.2 and was sterilized by autoclaving at 15 lb. sq. in. for 20 min. The indicator was 0.04% chlorophenol red. As the colour of an ethanolic solution was found to deteriorate when stored, the solid was dissolved in 1 ml. of ethanol and added to the medium immediately before tubing and sterilization.

Thirty 'carbohydrates' were used and 2% solutions, sterilized by Seitz-filtration, added to tubes of the basic medium to give a final concentration of

0.2 %. Before inoculation the tubes were incubated for 24 hr. to check sterility. Inoculated tubes were incubated for 7 days and readings taken on the 1st, 2nd, 4th and 7th days.

Although conflicting results by earlier workers have thrown some doubt on the value of the fermentation tests, they have given clear-cut results in this work and confirm the findings of Rogosa *et al.* (1953). The basal medium supported good growth in the presence of added sugar, and a 2 % inoculum was used. Since the inoculum was large, false positive results given by traces of acid in the inoculum had to be avoided by using an indicator with a low pH range; chlorophenol red (pH 4.8–6.4) was selected.

Bile salt tolerance. Tomato glucose broth of the following composition—tomato juice 10 %, Neopeptone 1.5 %, glucose 2 %, sodium chloride 0.5 %, Tween 80 0.1 %, Yeastrel 0.6 %, and soluble starch 0.05 %—was prepared, and to one-half of this basal medium 2 % sodium tauroglycocholate was added, the other half serving as a control. Both the control and the bile medium were filled in 5 ml. amounts into $\frac{1}{4}$ oz. McCartney bottles and sterilized for 20 min. at 15 lb. The inoculated bottles were incubated at 37° and read after 24 hr.

NaCl tolerance. Tests for NaCl tolerance were carried out in exactly the same way as those for bile tolerance, except that 2 % sodium chloride was substituted for sodium tauroglycocholate.

Starch tests. The most suitable medium for this test was found to be: Neopeptone 1.5 %, sodium chloride 0.5 %, Tween 80 0.1 %, Yeastrel 0.6 %, galactose 0.5 %, soluble starch 0.2 % and agar 2 %. It was noticed that the presence of glucose diminished the hydrolysis of starch; the addition of a carbohydrate, however, greatly stimulated the growth of lactobacilli, and galactose, which was not found to affect starch hydrolysis, was added to the medium. The cultures were streaked on the surface of plates of this medium and incubated in an atmosphere of 90 % hydrogen + 10 % (v/v) carbon dioxide at 37° for 3 days. They were tested for hydrolysis of starch by flooding the plates with a solution of iodine in potassium iodide.

RESULTS

The results of these tests, with the exception of the microtests, allow the separation of 78 of the 94 organisms studied into three well-defined groups (Table 2). These are *Lactobacillus acidophilus*, *L. bulgaricus*, and a further group with reactions intermediate between the other two. It is also possible, using these tests in conjunction with those described by Briggs (1953*b*), to separate these organisms from others strains of lactobacilli. Of the remaining 16 strains, a further 5 were identified as *L. lactis* and 2 as *L. fermenti*; 9 were not classified, but it is, of course, possible that these belong to other species which have not yet been examined in detail.

The microtests were found to be unsuitable for classifying these organisms since the results were not reproducible. It is possible that failure was due to the fact that it is very difficult to prepare standard cell suspensions of lactobacilli, for the following reasons. The suspensions used in this work were of standard

optical density, but, because of differences in size of the cells, they did not necessarily contain approximately equal numbers, nor were the proportions of dead and living cells constant. The cells for these tests are harvested and tested at a certain stage in their growth, unlike an inoculum in cultural tests, which continues to multiply during the test. Standard times and temperatures of incubation do not always produce suspensions which are identical, a slight variation in a batch of medium, for instance, may cause a slower rate of growth, and consequently give a suspension of different composition from that of a rapidly growing culture, although both have been incubated under identical conditions.

Table 2. *Differentiating physiological characteristics of the three groups.*

	<i>Lactobacillus acidophilus</i>	Intermediate	<i>Lactobacillus bulgaricus</i>
No. of strains in group	29	15	34
Growth at			
45°	+ (86 %)*	+	+ (85 %)
48°	—	+ (60 %)	— (76 %)
Tolerance of 2 % NaCl	+	+	—
Tolerance of 2 % Bile	+ (83 %)	—	—
Starch hydrolysis	+ (59 %)	—	—
Carbohydrate fermentations†			
Glucose	+	+	+
Galactose	+	+	+ (85 %)
Lactose	+	+	+ (94 %)
Fructose	+	+ (73 %)	+ (74 %)
Mannose	+	+	+ (91 %)
Dextrin	+ (83 %)	+	—
Maltose	+ (89 %)	+	—
Trehalose	+ (72 %)	— (60 %)	—
Amygdalin	+	—	—
Cellobiose	+	—	—
Salicin	+	—	—
Sucrose	+	—	—
Melibiose	+ (62 %)	—	—
Raffinose	+ (62 %)	—	—
Glycogen	— (57 %)	—	—

* The figures in parentheses indicate the percentage of strains giving the specified reaction.

† The following compounds were not fermented by any strains: adonitol, aesculin, arabinose, dulcitol, erythritol, glycerol, inositol, inulin, mannitol, melezitose, rhamnose, sorbitol, sorbose, starch and xylose.

LACTOBACILLUS ACIDOPHILUS

There were 29 organisms in this group: 15 strains named *Lactobacillus acidophilus* and 2 *L. bulgaricus*, 11 isolated from parts of the mammalian gut or from faeces and one from the mouth. It can be seen in Table 2 (footnote) that fifteen 'sugars' were not fermented by any strain, nine were fermented by all strains and the remaining six gave variable results. Melibiose and raffinose were fermented by 62 % of the strains, and a strain positive to one was always positive to the other. It is of interest that those sugars which were not fermented by all strains were also those which showed differences in the results

of duplicate tests of the same organism. The differences were, however, comparatively infrequent, occurring in only 1.3% of the tests.

Omitting those sugars giving variable results, four of the remainder, amygdalin, cellobiose, salicin and sucrose, which were fermented by all strains in this group and by none in the other two groups, can therefore be selected for purposes of differentiation.

No strains in this group grew in the presence of 4% bile salt but the majority tolerated 2%; 5 strains would not grow in either concentration. All strains grew in 2% sodium chloride.

The test for hydrolysis of starch did not give clear-cut results, and was therefore of little value.

LACTOBACILLUS BULGARICUS

This group contains 34 organisms: 18 strains named *Lactobacillus bulgaricus* and 2 *L. helveticus*, 9 strains isolated from sour milk preparations or animals fed with these, 3 from présure and two were of unknown origin. Only glucose was fermented by all strains, although lactose, mannose and galactose were fermented by the majority. Fructose, which was unheated (since all sugars were sterilized by Seitz-filtration), was fermented by 74%. No acid was produced from the remaining sugars, which included maltose and sucrose.

All strains in this group failed to grow in either 2% bile salt or 2% sodium chloride, and none of them hydrolysed starch.

INTERMEDIATE GROUP

In this group there are 15 organisms: 4 strains named *Lactobacillus helveticus* and 2 *L. jugurt*, 6 isolated from présure and 3 from milk. All these strains fermented glucose, galactose, lactose and mannose, in common with the first group of organisms and most strains of the second. They did not, however, ferment the four sugars, amygdalin, cellobiose, salicin and sucrose, fermented by *L. acidophilus*. Dextrin and maltose were fermented by all 15 strains; *L. bulgaricus* strains and a few of the *L. acidophilus* strains were negative to these two sugars.

No strain in this group tolerated 2% bile salt, nor hydrolysed starch, but they all grew in 2% sodium chloride.

DISCUSSION

It has been shown that *Lactobacillus acidophilus* and *L. bulgaricus* are distinct species and using the test conditions described here, it is possible to distinguish clearly between them, and to define a further group with properties intermediate between the two. The bile salt and NaCl tolerance tests, which have proved of great value in the identification of the organisms in the work reported here, are not mentioned in *Bergey's Manual* (Breed *et al.* 1948) nor are three of the four sugars which gave clear results in the fermentation tests.

Other tests which were tried, but were later discarded, include the Voges-Proskauer test and a test for production of tyrosine decarboxylase, using the method described by Sharpe (1948).

Colonial appearance

It was not possible to use colonial appearance as a distinguishing characteristic because of its variability. In the course of this work it was found that surface colonies of these organisms were not always similar. A plate prepared from one culture often gave a mixture of colonies, varying in shape and size, and standardization of techniques did not prevent this. Serological examination of different colonies (Sharpe, 1955) confirmed that they were identical with the original culture.

Carbohydrate fermentation

Rogosa *et al.* (1953) described strains of *Lactobacillus acidophilus* with properties similar to those described here; however, mainly on the basis of a weak or slow reaction in a single sugar, they recognized four variant types. In the present work the cultures of *L. acidophilus* showing variable reactions to one or two sugars were classified together in a single group, and the cultures put into the intermediate group vary in a number of characteristics from the other two groups. It was felt that a number of similar characteristics would give groups that could be clearly and easily recognized with less risk of error than would occur when a single characteristic was used.

It is stated in *Bergey's Manual* that *Lactobacillus bulgaricus* produces acid from glucose, lactose and galactose, but that there are conflicting opinions on the value of fructose, maltose and sucrose. Rahe (1918), for instance, used only maltose for the classification since some of his strains fermented sucrose. Kulp & Rettger (1924) found that *L. bulgaricus* would ferment heated fructose but not the unheated sugar. In the work described here, however, two-thirds of the strains of *L. bulgaricus* produced acid from unheated fructose, but none fermented maltose or sucrose. These findings are similar to those of Sherman & Hodge (1940). These authors also say that some old cultures of *L. bulgaricus* acquire the ability to ferment maltose and sucrose after prolonged laboratory culture. None of the strains in our collection possesses this property, although some of them have been cultivated in the laboratory for many years.

Serology

Serological work on these organisms (Sharpe, 1955) has confirmed the identity of some of the strains. Twenty-three of the 34 *Lactobacillus bulgaricus* strains have been found to belong to the same serological group, 6 have not yet been identified serologically and the remaining 5 belong serologically to *L. lactis*. Other strains of *L. lactis* which have been examined show very different sugar fermentations from the organisms grouped as *L. bulgaricus*. Further light may be thrown on this problem when the remaining cultures of *L. lactis* in our collection are examined in detail. Eleven of the 15 strains in the intermediate group are also serologically related. Sera have not yet been prepared for the *L. acidophilus* group.

Growth temperatures

In describing groups I–III of lactobacilli, Briggs (1953*b*) stated that although they were clearly separated from the remaining groups, they were not readily distinguishable from each other (Table 1), an opinion confirmed by this work. The strains comprising groups I–III were found, in the work reported here, to be distributed differently between *Lactobacillus acidophilus*, *L. bulgaricus* and the intermediate group (Table 3), indicating that growth

Table 3. *Distribution of strains in Briggs groups I, II and III between Lactobacillus acidophilus and L. bulgaricus*

	No. of strains	<i>L. acidophilus</i>	Intermediate strains	<i>L. bulgaricus</i>	Unclassified
Group I*	34	25	0	6	3
Group II	16	4	0	8	4
Group III	20	0	6	12	2
Section 1	24	0	9	8	9

* Data from Briggs (1953*a*).

temperatures cannot be used to distinguish between these organisms, in spite of their value in separating them from other groups. Growth temperatures have been used for classification by a number of workers, including Tittsler, Geib & Rogosa (1947), Curran *et al.* (1933) and Sherman & Hodge (1940), with varying degrees of success, and it seems apparent that whereas they are not always reliable as the sole means of identification, they are useful as supplementary tests. Much of the conflicting evidence is due to the use of different methods. It is obvious, for instance, that a large inoculum of an active culture is more likely to grow at a higher temperature than a small inoculum of a weak culture, since the chances of selecting resistant cells are greater. It is also more likely that a culture will grow at a temperature nearer to its maximum in an incubator than in an accurately controlled water-bath, since frequent opening of the incubator can cause considerable lowering of the temperature for varying periods of time. Most cultures used in this work had been found by Briggs (1953*b*) to grow at 45° and of those that at first failed, some have now been found to grow, if a larger inoculum is used. None of the cultures of *L. acidophilus* grew at 48° but some of those in the intermediate and *L. bulgaricus* groups grew at that temperature.

The names *Lactobacillus acidophilus* and *L. bulgaricus* have been used for two of the species described in this paper. Each group includes a number of named cultures of the species and representative strains from the National Collection of Type Cultures, and the American Type Culture Collection. The results described here supplement the original descriptions (Luerssen & Kuhn, 1908; Moro, 1900) rather than differ from them, except for the optimum growth temperature of *L. bulgaricus*. This was stated by the original authors (Luerssen & Kuhn, 1908) to be from 45 to 50°. It is difficult to define the optimum temperature, which may, of course, vary with the medium used, but the cultures described here as *L. bulgaricus* had in most cases a maximum

temperature between 45 and 48°. It is probable that the difference lies in the different techniques used for ascertaining growth temperatures, and also in the length of time the cultures have been cultivated in the laboratory. Sherman & Hodge (1936) found that freshly isolated cultures of *L. bulgaricus* would grow vigorously at 55° but after a year the same culture would not grow at 50°.

According to the International Code of Nomenclature (Buchanan, St John-Brooks & Breed, 1948) it is not permissible to exclude the original types when re-defining a group, but in this case the original type strains are not in existence, and amplification of the definition, to indicate that older cultures may have a lower maximum temperature than fresh isolates, is all that is required. This seems a more rational solution than to rename all the cultures of *Lactobacillus bulgaricus* on the grounds that their lower maximum growth temperature excludes them from that group. It is also in accordance with Rule 17 of the Code. Descriptions of *L. acidophilus* and *L. bulgaricus* in *Bergey's Manual* may now be supplemented by the following:

LACTOBACILLUS ACIDOPHILUS

No growth at 20° or below, and no growth at 48° and above. Grows well at 37°.

Distinctive characters: Homofermentative. Grows in acid media. Ferments amygdalin, cellobiose, salicin and sucrose. Will not grow at 48°. Growth in 2% but not in 4% NaCl or bile salt.

LACTOBACILLUS BULGARICUS

No growth at 20° or below; maximum temperature for growth of fresh isolates may be 50° or above, but for older cultures it is generally between 45° and 50°.

Distinctive characters: Homofermentative. Does not ferment amygdalin, cellobiose, salicin or sucrose. Growth at 48° variable. Will not grow in 2% NaCl or bile salt.

The group with properties intermediate to those of *Lactobacillus acidophilus* and *L. bulgaricus* has not been named. There are two alternatives, the first that it is a variety of *L. bulgaricus*, since it resembles that species more closely than *L. acidophilus*, and the second that it is a distinct species. It is felt that further and more detailed examination of these cultures is required to determine whether the characteristics in which these strains differ from *L. bulgaricus* are sufficient to define a separate species.

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The Characteristics of *Lactobacillus plantarum*, *L. helveticus* and *L. casei*

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SUMMARY: The characteristics of 152 strains of lactobacilli were examined, and strains divided into three groups. *Lactobacillus helveticus* fermented inositol, sorbose, glycerol and rhamnose but not melibiose or raffinose, failed to grow in 4% bile salt, but gave a positive Voges-Proskauer reaction and a rapid acid clot in Yeastrel glucose litmus milk (Y.G.L.M.). *L. casei* would not ferment these sugars, nor tolerate 4% bile salt, rarely gave a positive Voges-Proskauer reaction, but produced a rapid acid clot in Y.G.L.M. *L. plantarum* always fermented melibiose, usually raffinose and sometimes rhamnose, but did not ferment inositol, sorbose or glycerol. Strains of these species tolerated 4% bile salt but gave a negative Voges-Proskauer reaction and produced an acid clot in Y.G.L.M. only slowly.

The identification of *Lactobacillus helveticus*, *L. casei* and *L. plantarum* has in the past been difficult and uncertain. These organisms have been confused with each other and with other species in the genus *Lactobacillus*.

Bacillus casei α , γ , ζ , and ϵ were isolated from Emmenthal cheese and differentiated from each other by von Freudenreich & Thoni (1904) using production of acid and gas, carbohydrate fermentation, growth temperatures and colonial appearance. Later, Orla Jensen (1919) reclassified *Bacillus casei* α as *Streptobacterium casei*, and *Bacillus casei* ϵ as *Thermobacterium helveticum*; he also described *Streptobacterium plantarum*. The thermobacteria were distinguished from the streptobacteria mainly by their higher growth temperatures and with some species, by the optical activity of the lactic acid produced. This separation of *S. plantarum* from *S. casei* was based on the production of inactive lactic acid by the former, and dextro-lactic acid by the latter. He stated, however, that strains of *S. casei* might produce mainly inactive acid on first isolation and that some of his strains of *S. plantarum* produced mainly dextro-lactic acid. Further characteristics such as 'as a rule prefers maltose to saccharose and lactose' do not help in the definition of the two species. Pederson (1936) gave a clear description of *Lactobacillus plantarum* but did not compare it with *L. casei* or *L. helveticus*.

Sherwood (1939) isolated 594 strains of lactobacilli from New Zealand Cheddar cheese and examined a number of them in detail. He divided them into *Lactobacillus plantarum*, *L. casei* and a group of strains with intermediate properties. Both species and the intermediate group were further subdivided on the basis of sugar fermentations, and the effect of salt and heat on acid production. It seems possible that the second group of *L. casei*, which have a higher growth temperature and ferment rhamnose, are strains of *L. helveticus*.

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Curran, Rogers & Whittier (1933) studied *Lactobacillus acidophilus* and found that by growth temperatures, morphology and carbohydrate fermentation, this species could be separated from another group of organisms thought to contain strains of both *L. casei* and *L. bulgaricus*. Pederson (1947), however, found some strains of *L. acidophilus* which produced dextro-rotatory lactic acid; in this and other respects they were similar to strains of *L. casei* and he concluded that they were rough and smooth variants of a single type. The higher maximum growth temperature of *L. acidophilus* was used to separate this species from *L. casei* by Sherman & Stark (1927).

In a study of 500 lactobacilli from oral sources, Rogosa, Wiseman, Mitchell, Disraely & Beaman (1953) recognized three varieties of *Lactobacillus casei*, each variety being divided into three or more groups, in some cases by fermentation of a single sugar. They also described three subgroups of *L. plantarum*, but did not define *L. helveticus*. They found no difficulty in distinguishing *L. acidophilus* from *L. casei*.

Briggs (1953) was one of the few workers to attempt the study of the genus as a whole, and, using their wide range of growth temperatures (15–48°) and salt (NaCl) tolerance, she placed strains of *Lactobacillus helveticus* in one group (group IV). *L. plantarum* and *L. casei*, with a much lower maximum growth temperature but the same salt tolerance, were put in another group (group VI). By these tests *L. plantarum* could not be distinguished from *L. casei*. *Bergey's Manual* (1948) does not give distinguishing characteristics for these species, apart from the type of lactic acid produced. This test does not appear to be very reliable (Orla-Jensen, 1919; Pederson, 1947; Harrison & Hansen, 1950), and although possibly useful in the detailed examination of a few cultures, is laborious for the identification of large numbers of strains (Sherwood, 1939; Briggs, 1953).

The aim of the present work was to find simple tests which, by adding to the known characteristics, would clearly distinguish between *Lactobacillus helveticus*, *L. casei* and *L. plantarum*.

METHODS

Cultures and maintenance. The 152 cultures examined in this work are those comprising group IV (25) and group VI (127) in the classification of Briggs (1953) (Table 1). These groups consisted mainly of strains of *Lactobacillus casei*, *L. plantarum* and *L. helveticus*, but included named strains of many other species; amongst them were *L. acidophilus*, *L. bulgaricus*, *L. fermenti*, *L. delbrueckii*, *L. lactis*, *L. arabinosus*, *L. odontolyticus* and *L. pentosus*. Strains isolated from Stilton cheese, silage and fresh and sour milk had also been classified in groups IV and VI and were included in this work.

The cultures were maintained and stored in the same media and under the same conditions as those described by Briggs (1953). The carbohydrate fermentation tests, and those for the tolerance of bile salt, were described by Wheeler (1955).

Voges-Proskauer reaction. The medium, consisting of Neopeptone 1.5%, glucose 2%, NaCl 0.5%, Tween 80 0.1% and Yeastrel 0.3%, had a final

pH of 6.8 and was tubed in 5 ml. quantities. The tubes were inoculated with 0.05 ml. of an actively growing culture and incubated at 30° for 4 days. The cultures were then tested by the method of Barritt (1936).

Action on Yeastrel glucose litmus milk. The production of acid and the coagulation of milk by these strains was studied in separated milk containing 0.3 % Yeastrel, 1 % glucose and 1.5 % of a 1 % solution of litmus (Y.G.L.M.). Tubes of this medium were inoculated with a 4 mm. loopful of a 48 hr. culture in Y.G.L.M. and incubated at 30°. The tubes were examined for acid and coagulation after incubation for 48 and 72 hr.

Table 1. *Physiological classification of the lactobacilli* *

	Gas from glucose	NH ₃ from arginine	Survival at		Growth at			Tolerance 4 % NaCl	No. of strains
			60° for 30 min.	65° for 30 min.	15°	45°	48°		
Group I	—	—	—	—	—	+	—	—	34
Group II	—	—	—	—	—	—	—	—	16
Group III	—	—	+	—	—	+	—	—	20
Group IV	—	—	—	—	+	+	+	+	25
Group V	—	—	+	+	—	+	+	—	82
Group VI	—	—	—	—	+	—	—	+	127
Group VII	+	+	—	—	+	—	—	+	55
Group VIII	+	+	—	—	—	+	—	—	31
Section 1	—	—	+	—	—	+	+	—	24
Section 2	—	—	—	—	—	+	—	+	11
Section 3	—	—	—	—	+	+	—	+	12
Section 4			Miscellaneous reactions						15

* After Briggs (1953).

RESULTS

Using these methods, 141 of the 152 strains could be identified; one of the remaining eleven was found to be *Lactobacillus brevis*, but the other ten could not be named. The 141 strains were distributed between three species. Twenty-three strains were *L. helveticus*, 35 *L. casei* and 83 *L. plantarum*; the properties of these species are shown in Table 2. All strains were homofermentative and were separated from other homofermentative strains by the tests described by Briggs (1953), i.e. *L. plantarum* and *L. casei* grew at 15° but were unable to grow at 45° or 48°, *L. helveticus* grew at all three temperatures, and with a few exceptions, strains of all three species tolerated 4 % NaCl (Table 1).

It can be seen (Table 2) that, omitting the sixteen sugars which gave positive or variable results and the four which gave negative results with all three species (Table 2, footnote), the remaining sugars allow clear differentiation of the three species. Further confirmation was obtained by the tests for tolerance of 4 % bile and the action of the organisms on Y.G.L.M.

LACTOBACILLUS HELVETICUS

Of the 23 strains in this group, 9 were received as *Lactobacillus helveticus*, 6 as *L. casei*, 3 as *L. acidophilus* and 3 as *L. delbrueckii*; 2 were oral strains and the remainder were isolated from yoghurt.

All strains of *Lactobacillus helveticus* fermented inositol, sorbose and rhamnose, the majority fermented glycerol, but none fermented melibiose, raffinose, arabinose, inulin, adonitol or xylose. No strains would grow in the presence of 4% bile salt, but all except one gave a positive Voges-Proskauer reaction. All strains gave an acid clot in Y.G.L.M. after 48 hr. incubation at 30°.

Table 2. *Differentiating characteristics of Lactobacillus plantarum, L. helveticus and L. casei*

	<i>L. plantarum</i>	<i>L. helveticus</i>	<i>L. casei</i>
No. of strains	83	23	35
Growth at			
15°	+	+	+
45°	—	+	—
48°	—	+	—
Tolerance of 4% bile salt	+	—	—
Voges-Proskauer reaction	—	+	—
Acid clot in Y.G.L.M.			
2 days	—	+	+
3 days	—	+	+
Carbohydrate fermentations†			
Inositol	—	+	—
Sorbose	—	+	—
Glycerol	—	+	—
Rhamnose	—	+	—
Melibiose	+	—	—
Raffinose	+	—	—
Arabinose	—	—	—
Xylose	—	—	—
Inulin	—	—	—
Adonitol	—	—	—

* The figures in parentheses indicate the percentage of strains giving the specified reaction.

† The following sugars were (a) fermented by all strains: amygdalin, cellobiose, glucose, galactose, lactose, fructose, maltose, mannitol, mannose, melezitose, salicin, sucrose, trehalose; (b) fermented by some strains of each species: dextrin, dulcitol, sorbitol; (c) fermented by none of the strains: aesculin, glycogen, erythritol, starch.

LACTOBACILLUS CASEI

The 35 strains found to belong to this species were 15 received as *Lactobacillus casei*, 3 as *L. plantarum*, 3 as *L. helveticus*, 1 as *L. lactis*, 1 as *L. brevis* and 1 as *L. delbrueckii*. The other strains were isolated from raw milk (2), sour milk preparations (4), cheese (2) and the mouth (3).

None of these strains fermented glycerol, rhamnose, melibiose, raffinose, arabinose or xylose, but the majority gave a positive Voges-Proskauer reaction. All strains produced acid and nearly all coagulated Y.G.L.M. in 48 hr. followed by reduction in 72 hr.

Strains of *Lactobacillus casei* were readily distinguished from *L. helveticus* by their inability to ferment rhamnose and, with few exceptions, their inability to ferment sorbose, inositol or glycerol or to produce acetylmethyl carbinol.

LACTOBACILLUS PLANTARUM

Eighty-three strains were identified; 22 of *Lactobacillus plantarum*, 5 of *L. arabinosus*, 2 of *L. casei*, 2 of *L. pentosus*, 1 of *L. fermenti*, 1 of *L. pentoaceticus* and 1 of *L. pastorianus*, and of the isolated strains, 26 were from Stilton cheese and 2 from Swedish cheese, 14 were from silage, 1 from fish silage, 2 from sour milk, 1 from raw milk and 3 from the mouth.

All strains fermented melibiose and the majority, raffinose; about half also fermented rhamnose and arabinose and few glycerol and xylose. Inositol, sorbose, inulin and adonitol were not fermented by any of the strains. With few exceptions, they tolerated 4% bile salt and gave a negative Voges-Proskauer reaction. Only a few of the cultures in Y.G.L.M. had produced acid in 48 hr., and although after 72 hr. all were showing acid, only 13% had clotted the milk.

Lactobacillus plantarum is distinguished from *L. helveticus* and *L. casei* by its ability to ferment melibiose and generally raffinose, and from *L. helveticus* by its failure to produce acid from inositol, sorbose, glycerol and often from rhamnose. It also differs from both species in its tolerance of 4% bile salt, and its slow growth and production of acid clot in Y.G.L.M.

DISCUSSION

The tests described in this work, supplemented by the growth temperature and gas production tests of Briggs (1953), show that the three species *Lactobacillus plantarum*, *L. helveticus* and *L. casei* are distinct and can be differentiated from each other and from all other species in the genus *Lactobacillus*. Other tests, including hydrolysis of starch in an agar medium, tolerance of 6% NaCl and production of amino acid decarboxylases, were discarded either because the results were variable or because they did not differentiate the organisms. The rapidity of acid production in milk was chosen in preference to the total percentage acid formed because it was found that a number of strains of *L. plantarum*, which produced acid only slowly, nevertheless gave a final acidity equal to that of *L. casei*.

It will be seen in Table 2 that in certain tests not all the strains in a species gave the specified reaction of that species. In general, however, only one atypical reaction was given by one strain; in other respects its characteristics agreed with those of the species. Since no two strains gave a number of similar atypical reactions, e.g. a strain of *Lactobacillus casei* differing from the majority in fermenting inositol, did not necessarily also differ in fermenting sorbose, no sub-division of any of the three species into varieties was made.

The strains included in the species *Lactobacillus helveticus* are, with two exceptions which could not be identified by the tests described here, those included by Briggs (1953) in her group IV, confirming that the wide range of temperatures at which strains of *L. helveticus* will grow is a valuable aid to their identification. The strains classified as *L. plantarum* or *L. casei* were those in Briggs group VI; nine strains of this group could not, however, be identified. Serological examination of the same strains by Sharpe (1955) also

confirms the identification of some of these organisms. All strains of *L. plantarum* belonged to the serological group *L. plantarum*, and 20 of the 23 strains of *L. helveticus* all possessed the same type antigen. Although no distinction could be made physiologically, the species *L. casei* was divided into two serological groups, and one of these groups, though possessing a different type antigen, had the same group antigen as strains of *L. helveticus*.

The results of the carbohydrate fermentation tests were highly reproducible and confirm that fermentation of melibiose is a characteristic distinguishing *Lactobacillus plantarum* from *L. casei* as was suggested by Orla-Jensen (1943). The results also show that *L. casei* can be distinguished from *L. acidophilus* by its ability to ferment mannitol and melezitose. Rhamnose, sorbose and inositol were consistently fermented only by strains of *L. helveticus*; *L. plantarum* strains were variable in their action on rhamnose, and a few strains of *L. casei* fermented sorbose and inositol. Orland (1950), in a study of the antigenic characteristics of a number of lactobacilli, found that one group possessing the same major antigen had similar fermentation reactions, in particular their ability to ferment rhamnose and sorbose. He thought all these strains were possibly of the same species although differently named, and this is supported by the work reported here. Some of his strains were included in the present study and the rhamnose-sorbose fermenters classified as *L. helveticus*. Although in general the carbohydrate fermentation tests confirm those of Rogosa *et al.* (1953) for *L. casei* and *L. plantarum*, it seems probable that the *L. casei* var. *rhamnosus*, which they describe, is really in fact *L. helveticus*. Its higher growth temperature and fermentation of rhamnose, sorbose and inositol identify it as a member of this species. Rogosa *et al.* (1953) did not describe a species *L. helveticus*.

Table 3.

<i>LACTOBACILLUS</i> <i>PLANTARUM</i>	<i>LACTOBACILLUS</i> <i>CASEI</i>	<i>LACTOBACILLUS</i> <i>HELVETICUS</i>
Growth at 15° but not at 45° or 48°.	Growth at 15° but not at 45° or 48°.	Growth at 15°, 45° and 48°.
Yeast glucose litmus milk: production of acid but rarely coagulation in 3 days.	Yeast glucose litmus milk: production of acid and generally coagulation in 2 days.	Yeast glucose litmus milk: production of acid and coagulation in 2 days.
<i>Distinguishing characters:</i> homofermentative. Ferments amygdalin, cellobiose, salicin, sucrose, melezitose and mannitol, also melibiose and raffinose and sometimes rhamnose. Does not ferment inositol, sorbose or glycerol.	<i>Distinguishing characters:</i> homofermentative. Ferments amygdalin, cellobiose, salicin, sucrose, melezitose and mannitol. Does not ferment melibiose, raffinose, rhamnose or glycerol, and rarely ferments inositol or sorbose.	<i>Distinguishing characters:</i> homofermentative. Ferments amygdalin, cellobiose, salicin, sucrose, melezitose and mannitol, also inositol, sorbose, rhamnose and glycerol. Does not ferment melibiose or raffinose.
Growth at 15° but not at 48°.	Growth at 15° but not at 48°.	Growth at 15° and at 48°.
Growth in 4% bile salt and generally in 4% NaCl.	Growth in 2% but not 4% bile salt, usually grows in 4% NaCl.	Growth in 2% but not 4% bile salt, usually grows in 4% NaCl.
Slow production of acid and clot in Y.G.L.M.	Rapid production of acid and clot in Y.G.L.M.	Rapid production of acid and clot in Y.G.L.M.

It is of interest to note that the few oral strains in our collection are distributed over a number of species. One was found to be a strain of *Lactobacillus acidophilus* (Wheater, 1955), 2 were *L. helveticus*, 3 were *L. casei* and 3 were *L. plantarum*, confirming the findings of Rogosa *et al.* (1953). Pederson (1936) suggested that *L. arabinosus* and *L. pentosus* were synonyms of *L. plantarum* and in the present work all strains of these two species were in fact identified as *L. plantarum*.

Since these tests have shown that *Lactobacillus plantarum*, *L. helveticus* and *L. casei* can be clearly identified, it is suggested that the descriptions in *Bergey's Manual* (1948) may now be supplemented as shown in the lists of distinguishing characters (Table 3).

I would like to thank Dr A. T. R. Mattick for his interest and advice in the course of this work, and Miss P. Burrows for technical assistance.

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Relationships between Viable Saccharolytic Bacteria in Rumen and Abomasum of the Young Calf and Kid

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SUMMARY: The abomasums of three very young calves and two kids, of which all, save one suckled kid, had been fed on milk by bottle, were found to be supporting large populations of lactobacilli of both homo- and heterofermentative types. The heterofermentative organisms in every instance corresponded in fermentation reactions to *Lactobacillus fermenti* and was also found in one calf's rumen. The single homofermentative strain isolated from a calf was very near to *L. acidophilus* in fermentation reactions. The kid abomasum homofermentative isolates all seemed to belong to a new and unusual variety of *L. acidophilus* (var. *caprae*). It was amyolytic, insensitive to aureomycin, and hydrolysed aesculin.

The rumen and abomasum of the young calf contained an atypical variety of amyolytic streptococcus, differing from the typical *Streptococcus bovis* of the adult rumen in being non-haemolytic and in fermenting mannitol. The typical rumen *S. bovis* is sometimes truly iodophilic. These rumen and abomasum streptococci and lactobacilli are probably not introduced via the milk.

In a previous paper (Mann & Oxford, 1954) we described some lactobacilli belonging to both homo- and heterofermentative types, which had been found occasionally in the rumen contents of young ruminating calves up to 8 weeks of age only. We now add a description of the Gram-positive cocci accompanying them in that situation with especial reference to the change from non- and atypical amyolytic streptococcal types to the typical rumen amyolytic streptococcus (as described by MacPherson, 1953) with increasing age of the calf up to 14 weeks. Although the abomasum contents of the adult ruminant are usually much too acid to support bacterial growth, this is not always so with the young calf, as was shown by Mann, Masson & Oxford (1954*b*) in an aureomycin-feeding experiment. It seemed worth while therefore to attempt to isolate aciduric bacteria (particularly lactobacilli) from the abomasum as well as the rumen of the 10-day old calf before rumination had become properly established, and thus to compare the respective microfloras before that of the rumen became specialized. We have also extended the investigation to young kids of the same age, since, as will be seen, their abomasum contents are quite acid from the beginning and unlikely to support any but extremely aciduric species.

METHODS

Examination of Gram-positive cocci isolated from the rumens of nine calves.

These cultures comprised the 69 isolates which, as mentioned by Mann & Oxford (1954), accompanied the lactobacilli described in that paper. They were obtained from the rumen contents of nine slaughtered calves belonging to three age-groups, with three calves in each group. These age-groups, subsequently

referred to as 1, 2 and 3, were approximately 5, 8 and 14 weeks old, respectively. The cultures were isolated from parallel series of bottle counts in which cellobiose and mannitol provided the respective carbohydrate substrates. They were studied in detail by the methods described by Mann, Masson & Oxford (1954*a*) for sheep rumen streptococci and micrococci.

Examination of bulk contents of rumen and abomasum of very young calves and kids. In each of the months of March, April and May 1954, a week-old Ayrshire bullcalf (hereafter denoted CF 1, CF 2 or CF 3) was purchased from a nearby farm and maintained in isolation for 3 or 4 days, before slaughter, on the usual ration of milk and 'starter' gruel. Although each calf had begun to nibble straw, in no instance was it really ruminating. Two very young kids (denoted K 1 and K 2), which had been born and bred in the Institute, were also slaughtered during the same period. K 1 had been maintained by bottle on goat milk from birth, while K 2 had been suckled continuously. It had, however, begun to nibble straw before it was slaughtered. In each case the rumen and abomasum contents were separately worked up within an hour of slaughter, the weights, pH values, visible presence or absence of coagulated milk being noted. Direct smears were heat fixed and stained by Gram's method, chiefly to determine whether or not Gram-positive rods were present in numbers.

Isolation of lactobacilli from rumen and abomasum of the very young calf and kid. The 'bottle count' previously referred to was modified for this purpose as follows. Serial dilutions of rumen or abomasum contents, usually up to 10^{-6} or sometimes 10^{-8} , were made in sterile quarter-strength Ringer's solution; 1 ml. of each dilution was added to 15 ml. sterile molten (45°) tomato extract agar (pH 5.9) prepared according to the directions of Rogosa, Wiseman, Mitchell, Disraely & Beaman (1953). The carbohydrate added to the medium was glucose. The subsequent procedure was as described by Mann & Oxford (1954).

Isolation of streptococci from rumen and abomasum contents of a very young calf. Not all the colonies which developed in the bottle counts proved to be lactobacilli; some were streptococci. These latter were ignored in the case of the kid since there exists at present no background of knowledge concerning streptococcal types in the adult goat rumen. With one calf, however (CF2, from the abomasum of which no homofermentative lactic acid organisms were isolated) an attempt was made to compare the predominant streptococcal types in rumen and abomasum respectively by the methods of Mann *et al.* (1954*a*).

RESULTS

Classification of calf rumen streptococci according to age of calf

It was possible to isolate streptococci in pure culture from the rumen of eight of the nine calves in the three age-groups referred to. The one exception, the oldest calf, yielded only micrococci (see below). Some 15–20 rumen streptococcal isolates were obtained in all from each age-group. Only one-third of these were amylolytic in age-group 1, while more than two-thirds were amylolytic in each of the older age-groups. Some of these isolates were iodophilic, i.e.

gave colonies on starch or maltose agar after 4 days incubation which became intensely blue-black when the plate was flooded with Lugol's iodine. These interesting rumen streptococci, the first of their kind to be isolated in pure culture, will be reported upon in a subsequent paper.

It was probable that all these rumen streptococci belonged to Lancefield's serological group D. Eleven isolates taken at random, including representatives from every calf age-group, were grouped by Fuller's (1938) formamide method. No extract reacted with Lancefield's group B antiserum; all save one reacted with group D antiserum. The one exception belonged to the middle age-group, but was not regarded as significant because three other isolates from this group reacted.

Although several amyolytic streptococci belonging to group D were isolated from age-group 1, they were not identical with the typical rumen amyolytic streptococcus of MacPherson (1953) which was, however, frequently obtained from the older calves' rumens. They rather resembled the atypical rumen amyolytic streptococcus encountered by Mann *et al.* (1954*a*) in the sheep's rumen. This atypical organism was not however encountered at all in calf age-group 3. It differs from the typical form chiefly in being always non-haemolytic, in giving larger and whiter colonies, and in fermenting mannitol. The fermentation and other reactions of 58 calf rumen streptococcal isolates are summarized in Fig. 1, in which the respective calf age-groups are also taken into account.

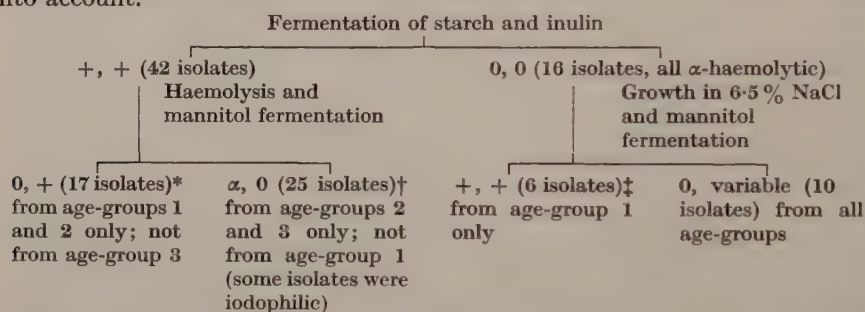


Fig. 1. Classification of 58 group D streptococcal isolates from calf rumen, according to fermentation reactions, and age of calf. + = fermentation or growth where appropriate; 0 = no fermentation or no growth; α = α-haemolysis.

* 'Atypical' rumen amyolytic streptococcus.

† 'typical' rumen amyolytic streptococcus.

‡ *Streptococcus faecalis*.

Micrococci from the rumen of a calf in age-group 3

In this one instance, both the cellobiose and mannitol bottle counts yielded preponderantly staphylococcal colonies. Eight isolates, all coagulase-negative, were studied in detail; six were both VP and MR positive and probably belonged to subgroup 2 of Shaw, Stitt & Cowan (1951). The remaining two isolates reduced nitrate, were MR positive and VP negative, and might be placed in subgroup 3 of the above classification. It is of interest that all eight isolates were urease-positive (cf. Mackay & Oxford, 1954).

*Properties of rumen and abomasum contents from three
very young calves and two kids*

As the results summarized in Table 1 show, the rumen and abomasum contents of the calves were in general more like each other than was the case with the kids. The rumen was relatively quite small in the supposedly non-ruminating kid; nevertheless, its contents had the 'adult' rumen pH value, and also the appearance and smell of the latter, and was singularly free from coagulated milk. Although the kid abomasum was in each case at pH 4, nevertheless the viable lactobacillus count was quite considerable, and comparable with that of the rather less acid calf abomasum (see Table 1).

Table 1. *Characteristics of rumen and abomasum contents of 10-day
and 11-day calves and kids*

Calf (CF) or kid (K) reference no.	Age of animal (days)	Rumen-reticulum contents			Abomasum contents		
		Weight (g.)	pH value	Viable 'lacto- bacillus + strepto- coccus' count (per μ g. rumen contents)	Weight (g.)	pH value	Viable 'lacto- bacillus + strepto- coccus' count (per μ g. aboma- sum contents)
CF1	11	1249	5.7	+	704	5.2	+
CF2	11	365	6.0	29*	530	5.5	> 100
CF3	10	579	5.8	> 100	616	4.3	> 100
K1	10	20	6.1	> 100*	210	4.0	13†
K2	11	61	6.7	> 1000*	186	4.0	> 1000†

* Chiefly streptococci; † chiefly lactobacilli; + = numerous, but no exact count made.

*Presence of lactobacilli as revealed by direct smears from rumen and
abomasum contents of calves and kids*

Gram-positive rods were always abundant in smears from the abomasum. They were usually long, often curved and were accompanied, as in the pure cultures of lactobacilli obtained from this situation, by degenerate Gram-negative forms of similar morphology. The rumen picture was always much more heterogeneous, with Gram-positive rods very much in the minority, and usually, when these occurred, they were not very similar to those in the corresponding abomasum smear, being smaller and more uniform in appearance. Gram-negative cocci were usually plentiful in rumen smears; Gram-positive cocci were rather less abundant, but still plentiful. It was concluded that although rumination had not become really established, the rumen microflora looked not very dissimilar from what would be expected for a more adult animal.

*Properties of Gram-positive rod isolates (lactobacilli) from rumen of calf CF1 and
from abomasum contents in three young calves and two kids*

Several isolates were made and purified in each instance. All were catalase-negative and did not reduce nitrate, i.e. were presumptive lactobacilli. Since it happened that all the heterofermentative, and (when isolated) all the

homofermentative lactic acid organisms isolated from any given rumen or abomasum were in every instance virtually indistinguishable from each other, it is not necessary to list the properties and fermentation reactions of the 32 isolates individually. Further examination showed that all the 19 heterofermentative isolates really belonged to one species, no striking differences existing. Thus, all fermented arabinose (but not xylose), galactose, glucose, lactose, maltose, melibiose, raffinose and sucrose but no other carbohydrate save fructose inconstantly. They did not hydrolyse aesculin nor attack hippurate. All grew at 45° but not at 16°. *Lactobacillus fermenti* rather than *L. brevis* seemed to be the species which would include all these heterofermentative isolates. The organism was isolated from all five abomasums examined and also from the most acid calf rumen, viz. that of CF1 (two isolates).

The homofermentative lactic acid organisms isolated from the two kid abomasums and also from the most acid calf abomasum (CF3) only, showed more diversity in fermentation reactions. The kid isolates were all very similar and had rather wider fermentative powers than the calf isolates which again were all very similar. The former (7 isolates in all, of which 5 were from K1) fermented cellobiose, dextrin, fructose, galactose, glucose, lactose, maltose, mannose, salicin, starch and sucrose with depression of pH to 3.9 in most instances; and also hydrolysed aesculin. The latter (6 isolates) did not ferment dextrin, maltose or starch, but unlike the kid isolates did attack trehalose in addition to the remaining sugars mentioned above with production of an acid pH value (3.9). Aesculin was not hydrolysed by the calf isolates. All these homofermentative isolates produced inactive lactic acid from glucose and tended to give rough colonies. All formed long rods and grew best under microaerophilic conditions; none grew at 16°. It was concluded that *Lactobacillus acidophilus* (cf. Rogosa *et al.* 1953) would cover the whole range of these homofermentative organisms, but that the kid strains, because being able to ferment starch and dextrin and to hydrolyse aesculin, might belong to a hitherto unnoticed variety in this species, which might be called *L. acidophilus* var. *caprae* since it was obtained from goat abomasum, concerning which situation no previous microbiological knowledge seems to exist. Unlike *L. acidophilus* from calf rumen both isolates of *L. acidophilus* var. *caprae* were quite resistant to aureomycin in glucose tomato extract broth, requiring instead of 4 µg./ml. at least 64 µg./ml. for complete inhibition of growth.

Streptococcal isolates from the rumen and abomasum of calf CF2

These comprised 13 isolates from rumen and 3 from abomasum. They were isolated by use of the rich tomato-extract agar medium (see Methods) instead of the rumen liquor agar of Mann *et al.* (1954*a*) and so are not strictly comparable with the 58 calf rumen streptococcal isolates previously classified in this paper. All 16 isolates were amyolytic; 6 from rumen and all 3 from abomasum belonged to the atypical rumen amyolytic streptococcus of Fig. 1. The remaining 7 isolates of which 5 were iodophilic, belonged to the typical rumen amyolytic streptococcus of Fig. 1.

Aciduric bacteria in fresh milk fed to the calves

When treated as for rumen contents, diluted and inoculated into glucose tomato-extract agar as previously described, the fresh milk yielded only streptococci in small numbers. Three isolates were tested and found not to be amylolytic; lactobacilli were never encountered.

DISCUSSION

The results make it clear that the abomasums of the very young calf and kid both support a large and diverse population of lactobacilli. Since, as Trautmann & Schmitt (1933) have shown, a back-flow of milk from abomasum to rumen may occur after feeding, and since food may pass direct to the abomasum in the young ruminant via the oesophageal groove, it is quite possible that the lactobacilli sporadically found in the calf rumen, both in this study and previously by Mann & Oxford (1954), may not normally be multiplying there but merely have been introduced from the abomasum where conditions are very favourable for their multiplication as long as the pH value remains at 4 or greater. The solitary calf rumen lactobacillus isolated did in fact belong to the same species (probably *Lactobacillus fermenti*) as found in the abomasum of the same calf (CF1).

None of the lactobacilli isolated in this study belong to species previously isolated from rather older calves by Mann & Oxford (1954). It is possible that it is a matter of chance which lactobacillus from the outside world finds a lodgement in the young ruminant's abomasum. The conditions there would be suitable for almost any microaerophilic lactose-fermenting lactobacillus with optimum temperature about 38° to multiply quickly. The results tend to indicate that such lactobacilli come from the solid food (straw?) or the mother's saliva, rather than milk. It is unlikely, for example, that the suckling kid (K2) could have taken in any lactobacilli with its mother's milk, yet it had a large population of these bacteria in its abomasum.

The present study also indicates that the typical rumen amylolytic streptococcus of the adult animal, although sometimes present in the young calf, does not become properly established until the rumen pH value is stabilized near neutrality. This seems not to happen until the calf is several weeks old (cf. Mann *et al.* 1954*b*). In the rumen of the very young animal, a somewhat different amylolytic streptococcus is to be found, which is possibly more tolerant of a smaller pH value. Both organisms, however, belong to Lancefield's group D and may be regarded as varieties of *Streptococcus bovis*, as defined in *Bergey's Manual* (1948).

We are indebted to Dr Margaret I. Chalmers of this Institute for placing the two kids at our disposal.

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ADDENDUM

After the completion of this work we noted that a strongly amylolytic and salicin-fermenting strain of *L. acidophilus* had very recently been isolated by Harrison & Hansen (1954) from blackhead liver lesions in turkeys. Their isolates, however, unlike our *L. acidophilus* (var. *caprae*), all fermented raffinose.

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The Purine Requirement of *Staphylococcus flavocyaneus*

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SUMMARY: *Staphylococcus flavocyaneus*, grown in chemically defined media, requires a purine; adenine, hypoxanthine, their respective ribosides and ribotides, 4-amino-5-imidazolecarboxamide or 4-amino-5-imidazolecarboxamidine were effective. Per mole, the ribotides (2', 3' or 5') were most efficient. Growth with effective purines is inhibited by guanine and certain unnatural purines. Inhibition of growth by purines may involve two mechanisms: one concerned with the synthesis of unnatural nucleic acid; the other with a crippling of ribose metabolism.

Guanine inhibits the growth of several purine-requiring organisms (Pennington, 1942; Fairley & Loring, 1949; Pontecorvo, 1950; Northam & Norris, 1951; Pomper, 1952). *Staphylococcus flavocyaneus* (Knaysi, 1942) was also inhibited (S. H. Hutner, unpublished observation); this inhibition has now been studied as an approach to the investigation of purine metabolism.

METHODS

The culture methods and measurements of growth responses used for *Staphylococcus flavocyaneus* (NCTC 7011) were similar to those used by Baker, Sobotka & Hutner (1953) for bacilli. Cultures were incubated at 37°. Although 3-4 days usually sufficed for growth with some compounds, cultures were allowed to grow for 10-14 days to allow the more slowly utilized but more active ribotides to be maximally used.

Stock cultures were grown on: K_2HPO_4 , 0.02 g.; Na_3 citrate. $2H_2O$, 0.05 g.; Na acetate. $3H_2O$, 0.03 g.; Na_2 succinate. $6H_2O$, 0.02 g.; Trypticase (Baltimore Biological Laboratory), 0.05 g.; yeast autolysate (Albimi Laboratories, Brooklyn, N.Y.), 0.1 g.; corn starch, 0.2 g.; agar, 1.6 g.; distilled water to 100 ml.; pH 7.0.

Purines and nucleic acid derivatives were obtained from commercial sources. The imidazoles and 2:6-diaminopurine were obtained through the generosity of Dr M. E. Balis of the Sloan-Kettering Institute for Cancer Research and the 8-azaguanine through the generosity of Dr G. H. Hitchings of the Wellcome Research Laboratories (Tuckahoe, New York, U.S.A.). The nucleic acid derivatives and analogues were tested for homogeneity with single-dimension ascending paper chromatography using the following solvent systems: Na_2HPO_4 . $12H_2O$, 5% (w/v) saturated with *iso*amyl alcohol (Carter, 1950), or butanol: glacial acetic acid: H_2O (4:1:5, v/v, Buchanan, Dekker & Long, 1950).

RESULTS

Nutrition

The basal medium (Table 1) was adequate for the growth and multiplication of *Staphylococcus flavocyaneus* upon addition of a purine. The main carbon and energy source was glucose, sucrose, or acetate; the combination of acetate + sucrose was optimal. Nitrogen could be supplied by NH_4Cl or L-glutamic acid (which also served as a supplementary carbon source); sulphur was provided by either $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, DL-methionine, or L-cystine. The optimal pH range was 7.5–8.0.

Table 1. *Basal medium for Staphylococcus flavocyaneus*

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.05 g.	Mo (as $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$)	0.8 mg.
KH_2PO_4	0.01 g.	Ca (as chloride)	1.0 mg.
NH_4Cl	0.05 g.	Trace metals solution*	1.0 ml.
Na acetate. $3\text{H}_2\text{O}$	0.4 g.	Distilled water to 100 ml.	
Sucrose	1.0 g.	pH 7.6–8.0	
L-Glutamic acid	0.5 g.		

* Trace metals solution 1.0 ml. contains the following:

Zn (as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$)	1.0 mg.	Cu (as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$)	0.2 mg.
Mn (as $\text{MnSO}_4 \cdot \text{H}_2\text{O}$)	1.6 mg.	Co (as $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$)	0.2 mg.
Fe (as $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$)	0.8 mg.	Ethylenediamine tetra-acetic acid	0.05 g.
B (as H_3BO_3)	0.4 mg.		

Table 2. *Compounds which satisfy the purine requirement of Staphylococcus flavocyaneus, and some which do not*

Utilized	Conc. range tested (mg./100 ml.)	Not utilized	Conc. range tested (mg./100 ml.)
Adenine*	0.01–100	Cytosine	2
Hypoxanthine*	0.01–100	Barbituric acid	2
Adenosine*	0.01–100	Orotic acid	2
Inosine*	0.01–30	5-Aminouracil	2
Adenosine-2'-phosphate*	0.01–100	Thymine	1–50
Adenosine-3'-phosphate*	0.01–100	Uracil	1–50
Adenosine-5'-phosphate	0.01–100	Guanine	0.01–5
Inosine-5'-phosphate	0.01–100	8-Azaguanine	0.01–50
Adenosine triphosphate	0.1–25	2:6-Diaminopurine.HCl	0.01–50
4-Amino-5-imidazole-carboxamide	0.01–84	Uric acid	1–50
4-Amino-5-imidazole-carboximidine	0.01–100	Allantoin	1–50
<i>Stimulatory</i> (in the presence of the above*)		Caffeine	1–50
Xanthine	0.01–3	Theophylline	1–50
		Theobromine	1–50
		L-Histidine	1–50
		4:5-Dicarboxyimidazole	2–20

Purine utilization

Adenine, hypoxanthine, their corresponding ribosides and ribotides, 4-amino-5-imidazolecarboxamidine ('amidine'), or 4-amino-5-imidazolecarboxamide ('carboxamide') satisfied the purine requirement (Table 2). The imidazoles correspond in configuration to adenine and hypoxanthine respectively (Fig. 1).

Utilization of purines and their derivatives was compared on a molar basis (Fig. 2). Growth appeared sooner with hypoxanthine, adenosine or inosine and lagged with adenine, the ribotides, amidine and carboxamide. Hypoxanthine, adenosine, and inosine satisfied the purine requirement at lower concentrations than did adenine or the ribotides (Fig. 2). At a concentration *c.* 1 μ mole/5 ml. adenine, hypoxanthine, or their respective ribosides fully satisfied the purine requirement. The ribotides (adenosine-2'-phosphate,

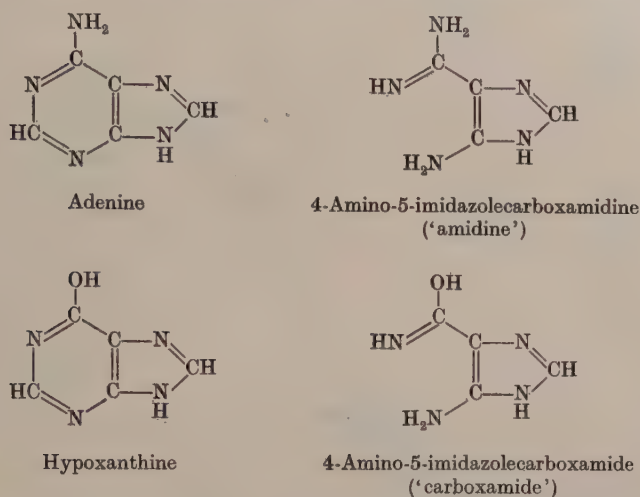


Fig. 1. Structures of adenine, hypoxanthine and the imidazoles used.

adenosine-3'-phosphate, adenosine-5'-phosphate, or inosine-5'-phosphate) allowed either no growth or poor growth at low concentrations; at higher concentrations ($> 1 \mu$ mole/5 ml.) these ribotides supplied the needed purine moiety better than did their respective ribosides or free bases. The latter inhibited at *c.* 50 μ mole/5 ml.

The response to 4-amino-5-imidazolecarboxamidine (Fig. 2) is characterized by a sharp peak; the abrupt drop in growth probably indicates toxicity. The response to 4-amino-5-imidazolecarboxamide was similar. The amidine allowed almost twice as much growth as the carboxamide at equal molarities, and also supported greater growth at the lower concentrations.

Inhibition of purine utilization

All the compounds which satisfied the purine requirement were inhibited by guanine and its analogues; inhibition curves for some of the compounds are shown in Fig. 3.

Hypoxanthine was chosen as the reference purine because low concentrations permitted good growth. Inhibition of hypoxanthine by guanine, guanosine and guanylic acid was in proportion to their guanine content. Growth was most diminished with ratios of guanine to hypoxanthine of *c.* 5.

Several purine analogues interfered with the utilization of hypoxanthine (Table 3) and also with adenine, adenosine, and adenylic acid (Table 4); the

guanine configuration seems essential for inhibition, for only compounds which contained guanine or had an amino group on carbons 2 or 6 were inhibitory. Guanine completely inhibited adenine utilization at the concentrations used in Fig. 8.

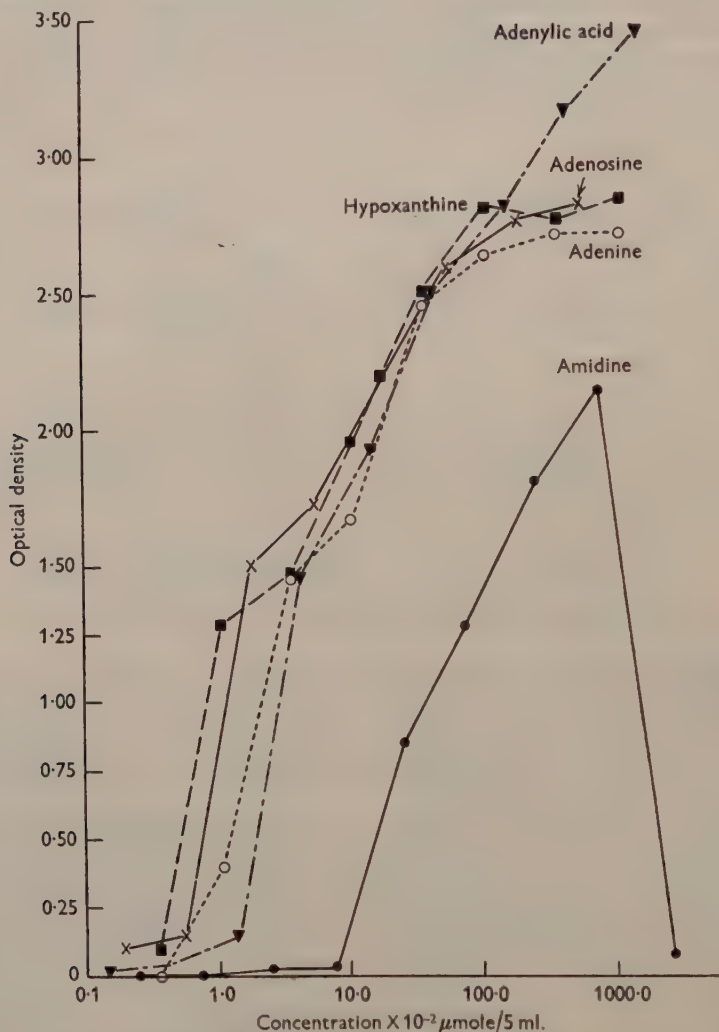


Fig. 2. Effect of purines and their derivatives on the growth of *Staphylococcus flavocyaneus*.

In this coccus the inhibition of growth by guanine and its riboside and ribotide is more effective than is inhibition by such purine analogues as 2:6-diaminopurine, 8-azaguanine, and isoguanine (Tables 3 and 4). The inhibition induced by these analogues (8-azaguanine was not tested) and guanine is partly or completely annulled by adenosine and adenylic acid (Table 4 and Fig. 3).

Attempts to overcome the guanine inhibition with known compounds other than nucleic acid derivatives were unsuccessful. The ineffective compounds included (alone or in various mixtures): *p*-aminobenzoic acid, folic acid, folinic

Table 3. *Inhibition of hypoxanthine utilization by purines, pyrimidines and their derivatives**

Compound	Range tested (mg./100 ml.)	Conc. of hypoxanthine (mg./100 ml.)				
		Degree of inhibition				
		0.01	0.05	0.1	0.5	1.0
Guanine	0.01-5	C†	C	C	C	P
Guanosine	0.02-100	—	C	C	C	—
Guanylic acid	0.02-25	—	C	C	P	P
8-Azaguanine	1-50	C	—	C	P	O
2:6-Diaminopurine	0.01-50	—	P	P	O	O
Isoguanine	1-10	—	P	—	P	O
Uric acid	1-50	—	O	O	O	O
Thymine	1-50	—	—	O	O	O
Uracil	1-50	—	—	O	O	O
Caffeine	1-50	—	—	O	O	O
Theobromine	1-50	—	—	O	O	O
Theophylline	1-50	—	—	O	O	O

* The results are a composite of many experiments.

† C=complete inhibition of growth within range; P=partial inhibition of growth (less than one-half of control); O=no inhibition of growth; —=not tested.

Table 4. *Comparison of various inhibitors on effect of several compounds utilized by Staphylococcus flavocyaneus*

Inhibitor	Concentration range ($\times 10^{-2}$ μ mole/5 ml.)	Adenine Hypo- Adeno- Adenylic Concentration ($\times 10^{-2}$ μ mole/5 ml.)			
		1.9*	1.9	1.9	1.8
		Degree of inhibition			
Guanine	0.33-99	C†	C	P	C
Guanosine	0.33-99	C	C	C	C
Guanylic acid	0.33-97	C	C	C	C
Isoguanine	0.33-99	P	P	O	O
2:6-Diaminopurine.HCl	0.33-99	P	P?	O	O
Uric acid	0.37-112	O	O	O	O
Xanthine	0.33-99	S	S	S	S

* This concentration gives about one-third maximum growth.

† C=complete inhibition within range; P=partial inhibition; P?=very slight inhibition; O=no inhibition; S=growth stimulation.

acid, the other B vitamins, and the common amino acids. Tests with complex materials such as beef extract, a tryptic digest of casein, and yeast autolysate indicated that only the beef extract had an activity possibly greater than that accounted for by its purine content (determined with a Beckman DU spectrophotometer).

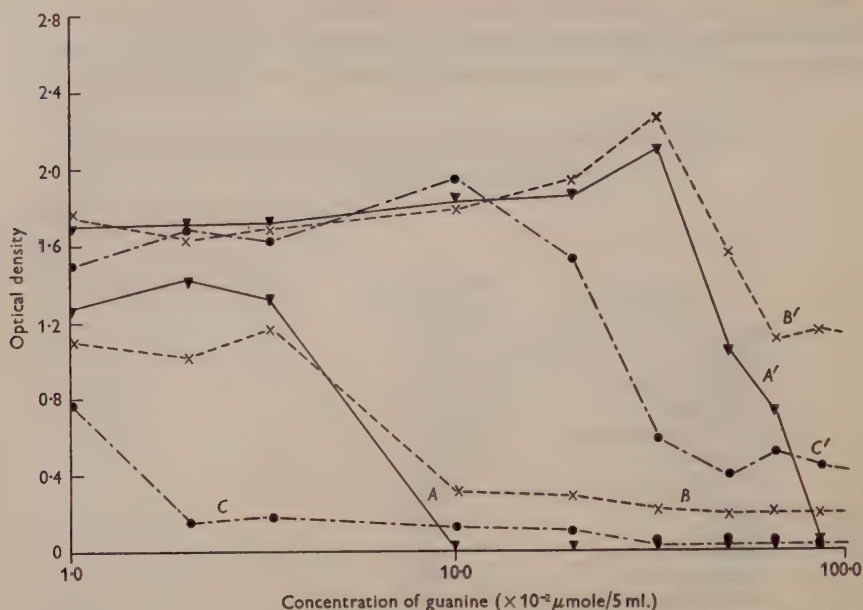


Fig. 3. Effect of several purines and their derivatives on the inhibition by guanine. (Adenine was ineffective at these concentrations.) Key: —, hypoxanthine, curve A, 1.9×10^{-2} $\mu\text{mole}/5$ ml.; curve A', 19×10^{-2} $\mu\text{mole}/5$ ml. ---, adenosine, curve B, 1.9×10^{-2} $\mu\text{mole}/5$ ml.; curve B', 19×10^{-2} $\mu\text{mole}/5$ ml. - · - · -, adenylic acid (2' and 3'), curve C, 1.8×10^{-2} $\mu\text{mole}/5$ ml.; curve C', 18×10^{-2} $\mu\text{mole}/5$ ml.

DISCUSSION

Utilization of purines and their derivatives. McNutt (1952) discussed the vagaries of the utilization of free purine bases, ribosides, and ribotides by micro-organisms. The pattern of purine utilization by *Staphylococcus flavocyaneus* has precedents. At present there seems to be no general hypothesis for the differential utilization of the purine bases and their derivatives. The superiority of the purine ribotides is not unusual (Fig. 2); several purine- and pyrimidine-requiring mutants of *Ophiostoma* spp. utilized the ribosides and ribotides better than the free bases (Fries, 1946); analogously, a strain of *Haemophilus parainfluenzae* used the pyrimidine ribosides and ribotides more effectively than free uracil (Herbst & Snell, 1949). *S. flavocyaneus* uses the three isomers of adenylic acid (adenosine-2'-phosphate, adenosine-3'-phosphate and adenosine-5'-phosphate) and inosine-5'-phosphate equally well. *Lactobacillus casei* used the 3' isomer of adenylic acid better than the 2' or 5' isomer (Balis & Elion, 1952). Whether the coccus converts the 2', 3', and 5' ribotides into a single member of this group or into another as yet unknown ribotide precursor of polynucleotides is unknown.

The imidazoles used here are of interest as possible precursors of nucleic acid purines (Greenberg, 1953). In the coccus, utilization of 4-amino-5-imidazolecarboxamidine (the adenine analogue) for growth is about twice as great as that of 4-amino-5-imidazolecarboxamide (the hypoxanthine analogue).

The amidine may be closer to the main pathway of purine synthesis than the carboxamide: it is more active and it provides a simpler pathway to adenine and one which bypasses hypoxanthine.

Inhibition of growth by purines and their derivatives. Growth inhibition by purines and their derivatives, natural and unnatural, has been reviewed by Roblin (1954) and by McNutt (1952). Guanine and other purines found in nucleic acids are inhibitors for several organisms (Pennington, 1942; Fairley & Loring, 1949; Fries, 1949). In some instances purine analogues such as 8-azaguanine, 2:6-diaminopurine or 6-mercaptapurine have offered promise in the inhibition of neoplasms (Gellhorn, Hirschberg & Kells, 1954; Law, 1950; Conference on 6-mercaptapurine, N.Y. Acad. Sci., 1954, various authors). Several hypotheses have been advanced to explain the interference with metabolism by purines and their derivatives. Fairley & Loring (1949), finding that guanine inhibited hypoxanthine but not adenine in a *Neurospora* mutant which required either adenine or hypoxanthine, suggested that guanine interfered with the amination of hypoxanthine. This does not explain the greater inhibition by guanine of adenine as compared with hypoxanthine in *Staphylococcus flavocyaneus*. Pennington (1942) thought that the inhibition by guanine of *Spirillum serpens*, which required adenine or hypoxanthine, was attributable to the need for a complex of two purine bases. Either two hypoxanthines or an adenine and a hypoxanthine molecule could make up the complex; guanine could be part of the complex only in the presence of either adenine or hypoxanthine. *Staphylococcus flavocyaneus* grows maximally with either adenine or hypoxanthine alone. There is no evidence that the addition of another base, i.e. adenine, hypoxanthine, or even guanine, increased growth appreciably; there was a small stimulation of growth in the presence of non-inhibitory amounts of guanine (Fig. 3).

Kalckar (1953) suggested two hypotheses to explain the growth inhibitions caused by purines and their derivatives: (1) unnatural purines divert pentose from synthetic pathways and thus interfere with nucleic acid synthesis; (2) unnatural purines form unnatural nucleic acids which then cannot function properly in their biological role and thus interfere with growth. Results with *Staphylococcus flavocyaneus* may support the first hypothesis. Here inhibition caused by 2:6-diaminopurine or isoguanine is completely annulled by adenosine and adenylic acid while the guanine inhibition is reversed only partially at the concentration of purine used (Table 4). Ribose-containing purine derivatives are exceptionally effective in overcoming the inhibition by guanine (Fig. 3). In recent experiments, D-ribose overcame inhibition by guanine in the presence of growth-promoting purine; further investigations are in progress. At low concentrations (1.8 or 1.9×10^{-2} $\mu\text{mole/5 ml.}$) of hypoxanthine, adenine and adenylic acid, growth is completely inhibited at the concentrations of guanine tested, while at a molar equivalent of adenosine growth is only partially inhibited. At 10 times the preceding concentrations adenine and hypoxanthine are still unable to annul the inhibition while adenylic acid partially annuls and adenosine almost completely annuls the inhibition of growth by guanine. The ribose-containing compounds may annul the blocking

of ribose utilization. The low solubility of guanine precludes trials of higher concentrations. The effectiveness of ribosides in overcoming inhibitions by purines and their analogues is in keeping with their effectiveness in suppressing the mutagenic activity of various purine analogues toward *Escherichia coli* (Novick & Szilard, 1952).

Kalckar's second hypothesis is supported indirectly by the isolation of ribonucleic acid which contains 8-azaguanine from *Escherichia coli*, *Staphylococcus aureus*, and both normal and tumour tissue of mice treated with 8-azaguanine (Lasnitzki, Matthews & Smith, 1954) and the demonstration by Matthews (1953) that 8-azaguanine is incorporated into nucleotides of tobacco mosaic virus. In addition, Jeener (1954) noted that the incorporation of thioracil into ribonucleic acid of tobacco mosaic virus resulted in fewer infective virus particles.

Inhibition by various purine analogues, especially by 8-azaguanine and 2:6-diaminopurine, is annulled by the purine bases. In several systems (psittacosis virus, Morgan, 1952; vaccinia virus, Thompson, Price, Minton, Elion & Hitchings, 1950; *Ophiostoma* sp., Fries & Panders, 1950; 'killer' action of *Paramoecium aurelia*, Williamson, Jacobson & Stock, 1952; cell division of plant tissues, Miller, 1953) inhibition by 2:6-diaminopurine is annulled by adenine. Inhibition of *Lactobacillus casei* and of *Tetrahymena geleii* by 8-azaguanine is annulled by guanine (Hitchings, Elion, Falco, Russel, Sherwood & Vanderwerff, 1950; Kidder & Dewey, 1949). In these systems the natural purine base may prevent the incorporation of the unnatural purine into nucleic acid.

As noted in the introduction, free guanine inhibits several purine-requiring micro-organisms besides *Staphylococcus flavocyaneus*. It is unlikely that guanine, a normal component of nucleic acid, forms abnormal nucleic acids when supplied exogenously. It would seem more likely that excess of exogenous guanine competes with required exogenous purines for pentose in nucleic acid synthesis, thus leading to a block in normal nucleic acid metabolism.

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Non-motile *Escherichia coli* O55, B5 Strains

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SUMMARY: A description is given of twelve non-motile *Escherichia coli* O55, B5 strains isolated during an outbreak of diarrhoea. Possible uses of such strains in serological practice and in epidemiological investigations are indicated.

Knowledge of the *Escherichia coli* group has been considerably extended by the studies of Kauffmann (see review, 1947), Vahlne (1945) and Knipschildt (1945, 1946) upon their antigenic structure. They used the term 'K antigens' to comprise the A (capsular) and L and B (thermolabile, surface) antigens; strains possessing K antigen were not agglutinated in the living state by homologous O antiserum and were described as O-inagglutinable (Kauffmann, 1944). B antigens differed from L antigens in that their agglutinin-binding power was not destroyed by heating; treatment of an OB antiserum with an O suspension of the homologous strain resulted therefore in the absorption of both the O and B agglutinins (Knipschildt, 1945). The O (somatic) antigens were shown to be thermostable and to resist heating at 100°.

Giles, Sangster & Smith (1949) reported the isolation from infants with diarrhoea of a specific serological type of *Escherichia coli* which they termed the β variety. Subsequently, Kauffmann & Dupont (1950) found that such strains possessed the antigens O 55 and B 5; their H antigens have been reported as types 2 (Laurell, 1952), 4 (Grönroos, 1954), 6 (Kauffmann & Dupont, 1950), 7 (Krepler & Zischka, 1952), 11 and 21 (Le Minor, Le Minor, Nicolle & Buttiaux, 1954).

The present paper gives an account of twelve non-motile strains of *Escherichia coli* O55, B5. They were isolated by Dr K. B. Rogers, in 1952, during an outbreak of infantile diarrhoea in a residential nursery in Birmingham. Nine of the strains were derived from young children, one from a nurse and two from dust. Three strains were selected for more detailed investigation and have been designated *E. coli* 886 (from a child), 899 (from a nurse) and 916 (from dust).

METHODS

Serological and biochemical tests. O and OB antisera were prepared with a representative *Escherichia coli* O55, B5, H6 strain, designated β 80; an OB antiserum with one of the test strains, *E. coli* 886; and antisera, for H agglutinin testing, with *E. coli* strains devoid of O55, B5 antigens, namely, Bi 7455/41 (O43, K?, H2), 5680 (O111, B4, H4), A20a (O2, K1, H6), and U5/41 (O1, K1, H7). In addition, two antisera were kindly provided by Dr J. Smith

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for use in testing for H reactions; they were prepared with *E. coli* Su 4321/41 (O13, K11, H11) and U11a/44 (O8, K?, H21).

Particulars of the preparation of *Escherichia coli* antisera, of their examination for certain non-specific agglutinins, and of the serological and biochemical methods used in testing *E. coli* strains have already been reported (Wright & Villanueva, 1953*a, b*).

Tests for motility. Each of the twelve strains under investigation was subcultured into two Craigie tubes (Craigie, 1931) containing semi-solid nutrient agar (0.3% agar content), one tube being incubated at 37° and the other at room temperature. The progress of the growth was observed daily. Examination of the outer growth for motility was made on the day when it was first observed on the surface of the medium and was repeated on each of the two subsequent days. Each Craigie tube culture was transplanted serially through a second and then a third Craigie tube; these were incubated under the same conditions as the first and were examined for motility in the same way.

In addition, strains 886, 899 and 916 were subcultured daily for 12 days in semi-solid nutrient agar stabs, one series with each strain being incubated at room temperature and the other at 37°. Each culture was examined daily for motility.

Tests for motility were carried out by the microscopic examination of hanging-drop suspensions in normal saline.

Staining for flagella. Stained preparations of strains 886, 899 and 916 were examined for the possible presence of flagella. Two 18 hr. MacConkey agar stab cultures and two 6-8 hr. nutrient broth cultures of each strain were tested, one of each duplicate set having been incubated at room temperature and the other at 37°. O55, B5 strains having H antigens 2, 6 or 7 were cultured in parallel, and on each slide prepared for the staining of a test strain one or other of these motile cultures was included as a control.

Mordant was freshly prepared by mixing 20 ml. 10% (w/v) aqueous tannic acid with 20 ml. saturated aqueous potassium alum and then adding 2 ml. 10% (w/v) ferric chloride; after standing for 30 min., it was filtered through a Whatman no. 1 filter-paper. A little growth from each solid medium culture was transferred to 4 ml. distilled water and the suspension allowed to stand for 5 min.; each broth culture was centrifuged and the deposit also treated in this way. A loopful of each suspension was spread on a microscope slide and left to dry in air. Mordant was applied to the slide preparation for 3-5 min., washed off with distilled water and the slide gently blotted to remove excess water. Steaming Fontana's silver nitrate solution was flooded on to the slide and left for 3-5 min. The stain was then washed off with distilled water, the slide gently blotted, dried in air, mounted in Canada balsam and examined immediately under the oil-immersion lens.

RESULTS

Serological tests

O-inagglutinability and B antigen tests. β 80 O antiserum at dilutions higher than 1/80 did not agglutinate living suspensions of the twelve strains under investigation; it agglutinated to a titre of 1/3200 their steamed suspensions.

The strains were thereby shown to be 'O-inagglutinable', indicating that each possessed a thermolabile surface antigen (see Kauffmann, 1944). That this antigen was of the B kind was demonstrated by the results of the following test. β 80 OB antiserum 1/25 was absorbed with O suspension of each of the strains 886, 899 and 916, and the absorbed antiserum was tested with saline suspensions of β 80 and of the three strains used for the absorption. In each instance, the absorption of both the O and B agglutinins was complete.

Tube-agglutination tests. 886 OB antiserum agglutinated OB suspensions of β 80 and of the twelve strains under test to a titre of 1/800. It agglutinated O suspensions of all these strains to a titre of 1/6400. It gave no agglutinative reactions with strains having α -antigen of Stamp & Stone (1944), β -antigen of Mushin (1949), a non-specific O antigen (strain 'Straughan') or Vi antigen.

β 80 OB antiserum agglutinated OB suspensions of β 80 and of the twelve strains to a titre of 1/400.

β 80 O antiserum agglutinated O suspensions of β 80 and of the twelve strains to a titre of 1/3200.

Slide-agglutination tests. Both 886 and β 80 OB antisera gave prompt and complete slide agglutination of saline suspensions of β 80 and of the twelve strains.

Agglutinin-absorption tests. 886 OB antiserum 1/25, using OB antigens, (a) after absorption with β 80, was tested with this strain and with each of the strains 886, 899 and 916, and (b) after absorption with each of these three strains, was tested with β 80 and with the strain used for the absorption. Absorptions were carried out in the same way with (a) 886 OB antiserum 1/50, using O antigens, (b) β 80 OB antiserum 1/25, using OB antigens and (c) β 80 O antiserum 1/50, using O antigens. In each instance, absorption of the O or OB agglutinins, respectively, was complete.

Possible H antigens in test strains. Investigations were made for the possible possession of H antigens by the strains 886, 899 and 916. Accordingly, 8 and 18 hr. nutrient broth cultures and formol-saline suspensions from the surface growth of (a) Craigie tube cultures in semi-solid nutrient agar, and (b) MacConkey agar stab cultures were prepared. Each type of culture was incubated in duplicate at room temperature and at 37°. Tube-agglutination tests were carried out with these suspensions, using antisera prepared with *Escherichia coli* strains possessing H antigens of type 2, 4, 6, 7, 11 or 21 but devoid of O55, B5 antigens. No H agglutination reactions were observed at antiserum dilutions 1/50–1/12800. Conversely, 886 OB antiserum was tested for the possible presence of H agglutinins of types 2, 4, 6, 7, 11 or 21. *E. coli* strains possessing H antigen of one or other of these types, but devoid of O55, B5 antigens, were cultured in MacConkey agar stabs at room temperature for 18 hr. and formol-saline suspensions prepared from them. H agglutination reactions were not observed at antiserum dilutions 1/50–1/12800. In every series of tests, appropriate control tests of the H antisera and suspensions were included and they yielded results which demonstrated the activity of the reagents employed.

Tests for motility

The period taken for the growth of the twelve strains to progress through the Craigie tubes to reach the outer surface of the medium varied from 2 to 15 days. Inspection showed a characteristically non-motile type of *Escherichia coli* growth which extended up the outer side of the inner tube and across the surface of the medium but did not diffuse through it. Motile organisms were not observed in the microscopic examinations of the cultures.

The growth of strains 886, 899 and 916 throughout twelve daily passages in semi-solid nutrient agar stab cultures remained of the non-diffusive type and motile organisms were not observed by microscopic examination.

Flagella staining

Flagella were not observed in the stained preparations of strains 886, 899 and 916. The control preparations of motile O55, B5 strains possessing H antigens of types 2, 6 or 7 all showed well-marked flagella development.

Biochemical reactions

The twelve strains under test produced acid and gas within 24 hr. at 37° in arabinose, glucose, lactose, maltose, mannitol, and xylose, and in MacConkey broth at 44°; produced acid or acid and gas between 24 and 48 hr. at 37° in sucrose and between 48 and 72 hr. in rhamnose; failed to ferment dulcitol, salicin, inositol, or inulin or to liquefy gelatin. They were methyl red-positive and Voges-Proskauer-negative; they produced indole, reduced nitrates to nitrites, did not utilize citrate, and did not produce urease.

DISCUSSION

Motile strains of *Escherichia coli* O55, B5 have been isolated from infants with diarrhoea by a number of workers during recent years (Giles *et al.* 1949; Kauffmann & Dupont, 1950). In 1953, however, Le Minor described three non-motile *E. coli* O55, B5 strains received by her from Copenhagen and particulars of twelve non-motile *E. coli* O55, B5 strains isolated in England are reported here. The latter strains differed in their fermentative reactions from the non-motile strains of Le Minor (1953) and from motile *E. coli* O55, B5 strains possessing H antigens of types 2, 4, 6, 7, 11 or 21 (Kauffmann & Dupont, 1950; Wright & Villanueva, 1953*a*; Le Minor *et al.*, 1954; Grönroos, 1954); they formed, nevertheless, a biochemically homogeneous group. The twelve non-motile strains had been isolated from different individuals, or from dust, during an outbreak of diarrhoea in a nursery, and the uniformity of their biochemical activities suggests that such reactions may be of use in defining stable subtypes applicable to epidemiological investigations.

Two further points of interest in connexion with non-motile strains of *Escherichia coli* O55, B5 are their possible use in the preparation of OB anti-sera free from H agglutinin (Wright & Villanueva, 1954) and in H antigen transduction experiments such as those undertaken with salmonella strains by Stocker, Zinder & Lederberg (1953).

Finally the importance of method in the examination of *Escherichia coli* strains for their possible motility may be stressed. It is evident from the work of Stuart & Carpenter (1949) that the majority of *E. coli* strains are motile; careful investigation of strains under conditions optimal for the development of motility (Wright & Villanueva, 1953*b*) is therefore required before this characteristic can be excluded and a report of non-motility be made.

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The Metabolism of Sodium 2-Keto-D-gluconate by Micro-organisms

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SUMMARY: The ability of various bacteria, actinomycetes, yeasts and moulds to grow on a medium containing sodium 2-keto-D-gluconate as the major carbon source was investigated. The disappearance of 2-ketogluconate during growth was followed and the ability of washed unadapted cell suspensions to oxidize this substrate (as evidenced by O_2 uptake) was studied. Adapted strains were examined for the presence of a 2-ketogluconokinase; this enzyme was detected in organisms of the genera *Pseudomonas*, *Xanthomonas*, *Escherichia*, *Aerobacter*, *Paracolobactrum*, *Serratia*, *Erwinia*, *Bacillus*. Although some bacteria and yeasts consumed 2-ketogluconate during growth and washed cells were able to oxidize it (after an induction period) 2-ketogluconokinase activity was not detected in cell-free extracts, prepared from these organisms, namely: species of *Agrobacterium*, *Corynebacterium*, *Schwanniomycetes*, *Debaryomyces*, *Lipomyces*, *Candida*. Several moulds (chiefly Pyrenomycetes, Aspergillales and Fungi Imperfecti) displayed the same phenomena. A few strains grew weakly on the substrate; however, unadapted cells did not show uptake of oxygen. The remaining strains of bacteria, yeasts, moulds and all the actinomycetes were without activity on 2-ketogluconate.

The results in this paper substantiate the opinion that the 'direct oxidation' pathways of carbohydrate metabolism are very widespread and important among micro-organisms.

The enzymes of the pathway of carbohydrate metabolism which is called the Warburg-Dickens scheme, direct oxidation or hexose-monophosphate-oxidative route (HMP), have recently been extensively investigated. They have been detected and studied in many mammalian tissues (with exceptionally high concentrations in adrenal cortex, lactating mammary gland, lymphatic tissue and rat embryo in early stages of development); in plant tissues (various seeds, spinach, pea leaves) and in micro-organisms (yeast, *Escherichia coli* and *Aerobacter cloacae*). One of us (De Ley, 1953*a*, and unpublished results) recently showed it to be probable that this pathway is very common among bacteria.

The discovery of a new enzyme '2-ketogluconokinase' in adapted *Aerobacter cloacae* (De Ley, 1953*c*), followed by the isolation of a new phosphate ester, 2-keto-D-gluconate-6-phosphate (De Ley, 1954*a, b*) opened new possibilities to explore this field. It is the aim of the present paper to emphasize again the importance of the HMP-oxidative route in micro-organisms. We report here experiments on the O_2 uptake on, and the disappearance under aerobic conditions of, sodium 2-keto-D-gluconate by a series of bacteria, actinomycetes, yeasts and moulds, selected to give a fairly representative general view of microbial taxonomy. The strains which took up O_2 in the presence of 2-ketogluconate were examined for the presence of a 2-ketogluconokinase.

METHODS

Organisms used. We used strains from the personal collection of micro-organisms of one of us (J. de L.). This collection of bacteria is perhaps unique because it contains chiefly gluconate-consuming species. We only selected moulds which were able to use D-gluconate.

For bacteria and actinomycetes the nomenclature of *Bergey's Manual* (1948) was followed. A difficulty arose with some Enterobacteriaceae from the National Collection of Type Cultures (Colindale, London), since for these the nomenclature of the *Report of the Coliform Subcommittee* (1949) was followed, except for the strains which ferment lactose slowly, or not at all (*Paracolobactrum*; *Bergey's Manual*, 1948, p. 460). For this reason, both nomenclatures are used in Table 1. For yeasts the nomenclature of Lodder & Kreger-van Rij (1952) was followed.

2-Keto-D-gluconate. This substance was prepared by the method of Ohle & Wolter (1930) and by a microbiological method in which calcium gluconate was oxidized by a strain of *Pseudomonas putida* (Harsveldt, private communication). We prefer the latter method because of its simplicity and good yield.

Growth on and consumption of 2-keto-D-gluconate

Culture medium for bacteria. Concentrations (as %, w/v, final concentration): Difco yeast extract, 0.1; K_2HPO_4 , 0.5; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.025; $FeSO_4 \cdot 7H_2O$, 0.025. The pH value was brought to 8.5 and the solution boiled and filtered while still hot. After cooling, ammonium sulphate was added to 0.15 %. A concentrated solution of sodium 2-ketogluconate was separately prepared (from the calcium salt and sodium oxalate, with removal of calcium oxalate) and added to the above basal medium to have a final concentration of 0.9 %; the pH value was then adjusted to 7.2. The complete medium was sterilized by filtration, distributed in 5 ml. volumes and incubated for a few days at 30° to test sterility.

Culture medium for actinomycetes, yeasts and moulds. A modified Czapek medium was prepared consisting of (g.): $NaNO_3$, 0.2; K_2HPO_4 , 0.1; $(NH_4)_2SO_4$, 0.3; $MgSO_4 \cdot 7H_2O$, 0.05; Difco yeast extract, 0.2; KCl, 0.05; $FeSO_4 \cdot 7H_2O$, 0.001; sodium 2-ketogluconate (prepared as above), 0.9; dissolved in distilled water to 100 ml.; 1 ml. growth factor solution added; pH value adjusted to 5.6. This medium was sterilized by filtration, distributed aseptically in 5 ml. lots in test-tubes and incubated to test sterility. The growth factor solution contained (mg./100 ml. distilled water): 0.02, biotin; 4, calcium pantothenate; 20, inositol; 4, nicotinic acid; 2, *p*-aminobenzoic acid; 4, pyridoxin; 4, thiamine; 2, riboflavin.

Growth conditions. The cultures were placed on a shaking machine for maximal aeration in a constant temperature room at their optimal temperature, usually 30°, occasionally 20°, 25° or 37°. Bacteria were grown for 3 days, actinomycetes and yeasts for about 1 week and moulds for 2 weeks. Yeasts and moulds were also sometimes grown without shaking.

Determination of 2-ketogluconate consumption. The decrease of the 2-ketogluconate concentration was determined by the method of Luff & Schoorl (Schoorl, 1912). Uninoculated tubes of medium were always used as controls.

Oxidation of 2-ketogluconate by unadapted micro-organisms

Culture medium for bacteria (% w/v, final concentration): 0.5, Difco peptone; 0.25, Difco yeast extract; 1.5, agar; pH 7.2; in Roux flasks.

Culture medium for yeasts. Beer wort, 8° Balling; 1.5 % (w/v) agar; pH 5.6; in Roux flasks.

Warburg manometric experiments. The micro-organisms were grown at optimal temperature for 1–2 days, harvested, washed with physiological saline and centrifuged. They were resuspended in M/60 phosphate solution (adjusted to pH 7.2 for bacteria, 5.6 for yeasts) and shaken for 3 hr. at 30° to decrease the endogenous respiration.

The oxidation of 2-ketogluconate was studied in the Warburg respirometer at 30°. Each vessel contained: 1.4 ml. suspension of bacteria or yeasts (*c.* 25 mg. dry weight), 0.5 ml. 0.066 M-phosphate solution (adjusted to pH 7.2 for bacteria, 5.6 for yeasts); the side arm contained 0.1 ml. water or 0.1 M-sodium 2-ketogluconate. The pH value of the contents of the Warburg vessel was roughly measured with bromthymol blue after the experiments.

Preparation of cell-free extracts of adapted micro-organisms

Cultures. These were the same as those used to establish the consumption of 2-ketogluconate. Bacteria and yeast cultures were used when 1–2 days old, mould and actinomycete cultures when about 1 week old.

Cell-free preparations of 2-ketogluconokinase. (a) Grinding with alumina (McIlwain, 1948). After grinding for 3 min. in the cold, extraction proceeded with 0.05 M-Tris buffer [tris-(hydroxymethyl)-aminomethane; pH 7.4; Gomori, 1946] for 30 min. at 0°, followed by centrifugation at 0° in a Servall angle head centrifuge at 5000 *g* for 1 hr. The supernatant fluid was used as the enzyme preparation. This method, and the following one, had previously been used successfully to obtain soluble 2-ketogluconokinase from *Aerobacter cloacae*. We used this method with all the bacteria and yeasts, and also with *Monascus ruber* and *Aspergillus flavus*.

(b) Use of the Hughes block (Hughes, 1951). The micro-organisms were introduced into the block previously cooled to –20°. They were crushed after a few vigorous blows with the Denbigh fly press and suspended in either 0.05 M-Tris solution (pH 7.4) or 0.01 M-phosphate buffer (pH 7.4). Microscopical observation showed that nearly all cells were disrupted. Either the supernatant fluid after centrifugation, or the entire mass of debris was used. Some bacteria, yeasts, actinomycetes, *Neurospora sitophila* and *Aspergillus flavus* were subjected to this treatment.

(c) The Mickle disintegrator (Mickle, 1948). Three g. yeast or mould were mixed with 7 ml. 0.05 M-Tris buffer (pH 7.4) + 10 ml. of Ballotini glass beads and shaken in the Mickle apparatus at 0°. Every 10 min. microscopical colour

tests (Loeffler methylene blue and Gram stain) were carried out until at least 90 % of the cells appeared to be disrupted. The Ballotini were removed and the resulting suspension (including cell debris) was used as enzyme preparation.

(d) Mould tissue was ground in an all-glass homogenizer; 10 min. proved sufficient to disrupt nearly all the cells; spores were hardly attacked. The ground mass was suspended in Tris or phosphate buffer.

Paper chromatographic estimation of 2-ketogluconokinase activity (De Ley 1954a, b). The reaction mixture contained in 3 ml.: 0.3 ml. enzyme preparation; 24 μ mole Mg^{++} ; 30 μ mole NaF; 15 μ mole 2-ketogluconate; 24 μ mole ATP; 0.75 ml. 0.2 M-Tris buffer (pH 7.4). Every hour, for 3 hr., a sample (80 μ l.) was spotted, after de-cationization, on pre-washed Whatman paper no. 1 and chromatographed with a mixture of methanol (6 vol.) + concentrated ammonia solution (sp.gr. 0.880; 1 vol.) + water (3 vol.) at 4°. The developed chromatogram was sprayed with the o-phenylenediamine-HCl spray, which revealed the 2-keto-D-gluconate-6-phosphate specifically, as a violet spot.

RESULTS

The results obtained with organisms able to metabolize 2-ketogluconate are summarized in Tables 1-3. The strains of the following micro-organisms which were tested did not show any growth or disappearance of substrate when incubated with 2-ketogluconate, nor did washed unadapted cells consume O_2 in presence of this substrate in the Warburg apparatus:

Bacteria: *Pseudomonas fluorescens*, *P. cocovenenans*, *P. geniculata*, *P. ureae*; *Protaminobacter rubrum*; *Vibrio agar liquefaciens*, *V. comma*; *Azotobacter chroococcum*; *Rhizobium leguminosarum*; *Chromobacterium violaceum*; *Micrococcus pyogenes* var. *aureus*, *M. lysodeikticus*; *Alkaligenes faecalis*; *Achromobacter hartlebii*; *Proteus morgani*; *Bacillus subtilis*, *B. cereus*, *B. brevis*, *B. laterosporus*, *B. lentus*; *Mycobacterium phlei*.

Actinomycetes: *Nocardia opaca*, *N. caviae*, *N. lutea* and two undetermined; *Streptomyces erythreus*, *S. albus*, *S. griseolus*, *S. lavendulae* and one undetermined; *Micromonospora fusca*.

Yeasts: *Endomyces decipiens*; *Schizosaccharomyces octosporus*; *Endomycopsis fibuliger*; *Saccharomyces cerevisiae*, *S. cerevisiae* var. *ellipsoideus*, *S. pasteurianus*, *S. fragilis*; *Pichia farinosa*, *P. fermentans*; *Hansenula anomala*; *Hanseniaspora valbyensis*; *Saccharomycodes ludwigii*; *Nadsonia fulvescens*; *Nematospira coryli*; *Sporobolomyces roseus*; *Bullera alba*; *Candida mycoderma*, *C. utilis*, *C. crusei*; *Trigonopsis variabilis*; *Trichosporon pullulans*; *Rhodotorula glutinis*.

Moulds: *Absidia gracilis*, *A. orchidis*; *Cunninghamella elegans*; *Chaetomium globosum*; *Chaetothyrium javanicum*; *Elsinoe mangiferae*; *Fomes pinicola*; *Fusarium solani*.

The quantitative experiments on 2-ketogluconate consumption indicated the strains which had to be investigated further. A selection of strains with high degrees of activity was made in order to study the oxygen uptake of unadapted cells with 2-ketogluconate as substrate. A survey of these results

Table 1. *Decomposition of 2-keto-D-gluconate by various bacteria*

The number in brackets after the names of organisms gives the number of strains tested.

Organism	Degree of growth	Amount of 2-keto-gluconate decomposed (%)	O ₂ uptake of washed cells*	Formation of 2K6P by cell-free extract†
<i>Pseudomonas aromatica</i> (1)	Excellent	75	nt	+++
<i>P. aeruginosa</i> (1)	Good	71	nt	++
<i>P. putida</i> (1)	Good	34	nt	nt
<i>P. aromatica</i> var. <i>quercito-pyrogallica</i> (1)	Good	73	ind.	++
<i>P. fluorescens</i> (2)	Good	32-73	ind.	+
<i>P. ovalis</i> (1)	Good	40	nt	+++
<i>P. convexa</i> (1)	Good	75	nt	nt
<i>P. viscosa</i> (1)	Good	77	nt	nt
<i>Xanthomonas phaseoli</i> (1)	Weak	5	nt	+
<i>X. campestris</i> (1)	Weak	3	nt	+
<i>X. pruni</i> (1)	Weak	7	nt	±
<i>Agrobacterium tumefaciens</i> (1)	Excellent	73	ind.	-
<i>Corynebacterium simplex</i> (2)	Excellent	40-69	nt	-
<i>C. helvolum</i> (1)	Excellent	73	ind.	-
<i>C. poinsettiae</i> (1)	Good	15	nt	-
<i>Flavobacterium aquatile</i> (1)	Good	17	0	-
<i>Bacterium</i> (= <i>Escherichia</i>)				
<i>B. coli</i> I (3)	Weak-moderate	5-18	0	nt
<i>B. coli</i> I (1)	Excellent	90	nt	nt
<i>Bacterium coli</i> II (3)	Excellent	59-100	nt	++
<i>B. coli-anaerogenes</i> (1)	Weak	6	nt	nt
<i>B. intermedium</i> I (4)	Excellent	100	ind.	+++
<i>B. intermedium</i> II (1)	Excellent	100	nt	nt
<i>Bacterium</i> (<i>Aerobacter</i>)				
<i>B. aerogenes</i> I (8)	Excellent	100	ind.	+++
<i>B. aerogenes</i> II (1)	Weak		nt	nt
<i>B. aerogenes</i> II (2)	Excellent	100	nt	nt
<i>B. cloacae</i> (4)	Excellent	100	ind.	+++
<i>Paracolonobacterium aerogenoides</i> (1)	Excellent	100	ind.	+++
<i>Bacterium</i> (<i>Klebsiella</i>)				
<i>B. pneumoniae</i> (3)	Good	27-100	nt	nt
<i>B. rhinoscleromatis</i> (1)	Moderate	24	nt	nt
<i>B. ozaenae</i> (1)	Good	65	nt	nt
<i>Serratia plymouthis</i> (2)	Good	69	ind.	++
<i>Proteus vulgaris</i> (1)	Weak	13	0	-
<i>Erwinia carotovora</i> (1)	Moderate	11	0	+
<i>Bacillus subtilis</i> (1)	Weak	20	0	-
<i>B. cereus</i> var. <i>mycoides</i> (1)	Weak	17	nt	nt
<i>B. megaterium</i> (4)	Moderate-good	28-95	ind.	++
<i>B. circulans</i> (1)	Weak	31	nt	nt
<i>B. mesentericus</i> (1)	Moderate	33	0	-

* Oxygen uptake of washed cells: ind.=only after induction period; 0=no uptake; nt=not tested.

† Intensity of the spot of 2-keto-D-gluconate-6-phosphate-quinoxaline, after 3 hr. enzyme activity of cell-free extract: +++=very intense, nearly complete phosphorylation; ++=intense, excellent phosphorylation; +=weak phosphorylation; ±=very weak but certain enzyme activity; -=no kinase activity detectable (after 3 hr.).

Table 2. *Decomposition of 2-keto-D-gluconate by certain yeasts*

The number in brackets after the names of organisms gives the number of strains tested.

Organism	Degree of growth	Amount of 2-keto-gluconate decomposed (%)	O ₂ uptake of washed cells*	Formation of 2K6P by cell-free extract†
<i>Schizosaccharomyces pombe</i> (2)	Moderate	0-21	nt	nt
<i>Endomycopsis capsularis</i> (1)	Good	39	0	nt
<i>Saccharomyces delbrueckii</i> (1)	Good	14	nt	nt
<i>Schwanniomyces occidentalis</i> (2)	Excellent	64-91	ind.	—
<i>Debaryomyces hansenii</i> (1)	Excellent	74	ind.	—
<i>Hanseniaspora apiculata</i> (1)	Good	37	0	nt
<i>Lipomyces lipoferus</i> (1)	Excellent	81	ind.	—
<i>Torulopsis holmii</i> (1)	Good	13	0	nt
<i>Brettanomyces bruceellensis</i> (1)	Good	10	nt	nt
<i>Candida albicans</i> (1)	Excellent	100	ind.	—
<i>Kloeckera apiculata</i> (2)	Moderate-good	18-40	0	nt

* Oxygen uptake of washed cells; ind.=only after induction period; 0=no uptake; nt=not tested.

† As indicated by intensity of chromatogram spot for 2-keto-D-gluconate-6-phosphate-quinoxaline: — =no detectable kinase activity up to 3 hr.; nt=not tested. Compare Table 1.

Table 3. *Decomposition of 2-keto-D-gluconate by certain moulds*

Only 1 strain of each organism was tested

Organism	Degree of growth	Amount of 2-keto-gluconate decomposition (%)	Formation of 2K6P by cell-free extract*	Method of disintegrating cells and buffer used
ASCOMYCETAE				
<i>Neurospora sitophila</i>	Good	75	—	Mickle; Tris
<i>Nectria cinnabarina</i>	Moderate	100	nt	Hughes; phosphate
<i>Monascus ruber</i>	Good	76	—	nt
				Alumina; phosphate
<i>Aspergillus nidulans</i>	Good	82	nt	Mickle; Tris
<i>A. fumigatus</i>	Good	89	—	nt
<i>A. flavus</i>	Good	92	—	Homogenizer; Tris
				Alumina; Tris
<i>A. niger</i>	Good	88	nt	Hughes; Tris
<i>Penicillium chrysogenum</i>	Good	92	—	nt
<i>Penicillium</i> sp.	Good	90	—	Homogenizer; Tris
<i>Penicillium</i> sp.	Good	94	nt	nt
FUNGI IMPERFECTI				
<i>Cercospora beticola</i>	Average	74	nt	nt
<i>Helminthosporium sativum</i>	Good	92	nt	nt
<i>Monilia brunnea</i>	Weak	27	nt	nt
<i>Phoma betae</i>	Good	82	—	Homogenizer; Tris
<i>Dematium pullulans</i>	Weak	16	nt	nt

* As indicated by intensity of chromatogram spot for 2-keto-D-gluconate-6-phosphate-quinoxaline: — =no detectable kinase activity up to 3 hr.; nt=not tested. Compare Tables 1 and 2.

showed that the unadapted strains which were able to grow on 2-ketogluconate could be divided into two distinct groups with respect to oxygen uptake.

(i) Strains which consumed oxygen when 2-ketogluconate was substrate always did so after an induction period. The 2-ketogluconokinase of *Aerobacter cloacae* K 3 is an adaptive enzyme (De Ley, 1953c). Figs. 1 and 2 illustrate the kind of oxygen-uptake curve which was obtained.

(ii) The second group of organisms contained those which, although able to grow poorly or moderately on 2-ketogluconate and partially to decompose it, did not show an oxygen uptake in Warburg experiments with 2-ketogluconate as substrate. The moderate growth in these cases appeared to be due to the selection of mutants during growth.

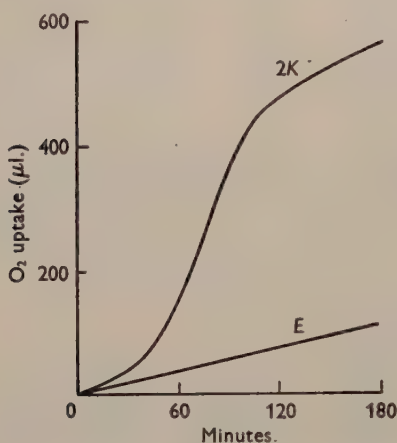


Fig. 1.

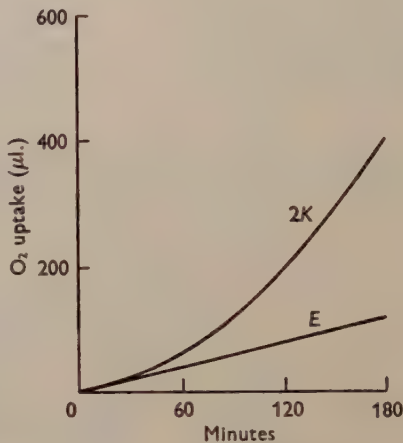


Fig. 2.

Fig. 1. The respiration of *Serratia plymuthicum* in presence of sodium 2-keto-D-gluconate (curve 2K); curve E: the endogenous respiration. For contents of the Warburg vessels, see text.

Fig. 2. The respiration of the yeast *Lipomyces lipoferus* in presence of sodium 2-keto-D-gluconate (curve 2K); curve E: endogenous respiration. For contents of the Warburg vessels see text.

The problem was investigated further by the study of the distribution of 2-ketogluconokinase in a carefully selected set of strains. A survey of these results shows that this soluble enzyme was present only in some of the bacteria examined; its absence from some 2-ketogluconate-metabolizing bacteria, yeasts and moulds was striking. Since we worked only with centrifuged extracts of alumina-ground cells in Tris buffer, it was possible that the enzyme in these strains was inactivated by this treatment or was bound to particles. We therefore used other disintegration and extraction methods (see Methods). Certain micro-organisms were disrupted by more than one method: *Corynebacterium helvolum* (alumina, Hughes block), *Candida albicans* (alumina, Hughes block, Mickle), moulds (see Table 3). We used both crude suspensions and the supernatant fluid obtained after centrifugation. We never observed

even the slightest spot of 2-keto-D-gluconate-6-phosphate on the chromatograms. This showed that the enzyme was not bound to particles. Since it is known that several enzymes require inorganic phosphate for normal activity, we substituted phosphate buffer for Tris, again however without result.

DISCUSSION

Enterobacteriaceae. Nearly all the members of this family are able to grow on 2-ketogluconate, to oxidize it and to form adaptively a 2-ketogluconokinase. All the Enterobacteriaceae tested grow well on gluconate (De Ley, 1953*a, c*). *Proteus vulgaris* seems unable to form this enzyme. Among the *Erwinia* the enzyme activity is very small, which explains the absence of O₂ uptake. Although the formation of 2-keto-D-gluconate-6-phosphate by adapted *Klebsiella* spp. was not investigated (because of its high virulence) the presence of this enzyme here may also be taken for granted. It has been shown that *Escherichia coli* (McNair Scott & Cohen, 1951) and *Aerobacter cloacae* (De Ley, 1953*a-c*) possess the enzyme system for the HMP-oxidative route. From the present results it may fairly be generalized that nearly all, if not all, the members of the family Enterobacteriaceae possess this metabolic pathway (with the possible exception of *Proteus*, *Salmonella* and *Shigella*; the last two are not yet investigated).

Aerobic Bacillaceae. We observed previously that many organisms of this family are able to use gluconate (De Ley, 1953*a*). Although several species are able to use 2-keto-gluconate, *Bacillus megaterium* is the only one which shows adaptive formation of a 2-ketogluconokinase. In a preliminary note De Donder (1952) showed that both *B. subtilis* and *B. megaterium* possess a TPN-linked gluconate-6-phosphate dehydrogenase. All these facts are in favour of the view that in some members of this family also an HMP-oxidative route is present, or at least a very similar one.

Pseudomonas and Xanthomonas. It was interesting to find in the Bacillaceae 2-ketogluconokinase, although it was irregularly distributed among the different species. Some *Pseudomonas* spp. contain a hexokinase (Entner & Doudoroff, 1952; Klein, 1953), whereas others do not (Wood & Schwert, 1953). Our results show that many *Pseudomonas* and *Xanthomonas* spp. have an enzyme system for phosphorylative carbohydrate metabolism, at least after the stage of the 2-ketogluconate formation. In this connexion it must be remembered that several authors agree (Entner & Stanier, 1951; Stokes & Campbell, 1951; Wood & Schwert, 1953, 1954) that the transformation: glucose → gluconate → 2-ketogluconate in *Pseudomonas* spp. occurs without the intervention of phosphorylated intermediates. It is now clear that *P. fluorescens* and *P. saccharophila* have yet another route of gluconate-6-phosphate decomposition, namely by splitting into triose-phosphate and pyruvate (Entner & Doudoroff, 1952; Wood & Schwert, 1954) presumably after the initial formation of 2-keto-3-deoxygluconate-6-phosphate. Paper chromatography revealed that both *Pseudomonas* and *Xanthomonas* formed an unidentified reducing substance during 2-ketogluconate metabolism.

Bacteria which consume 2-ketogluconate but do not possess a soluble 2-ketogluconokinase. One strain of *Agrobacterium tumefaciens* and four *Corynebacteria* spp. occurred in this group. The mechanism of 2-ketogluconate metabolism by these strains is obscure and is discussed later.

Yeasts and moulds. Table 2 shows that only four yeasts from the collection tested were able to consume O_2 when 2-ketogluconate was the substrate. This oxidation occurred only after the formation of some adaptive enzyme which was not the normal soluble 2-ketogluconokinase. This same property is also common among moulds; it seems to be found chiefly among members of the pyrenomycetes, Aspergillales and Fungi Imperfecti. It is now clear that these yeasts, moulds and the above-mentioned bacteria do not possess a normal soluble kinase, activated by ATP and Mg^{++} . The exact mechanism is still obscure. Several explanations are possible: e.g. the presence of a kinase which requires a different activator or coenzyme, or an entirely new and unsuspected mechanism. The suggestion that some coenzyme or activator is lacking seems justified when it is remembered that these intact adapted micro-organisms rapidly decompose 2-keto-gluconate, whereas the same cells, when disintegrated, leave it completely unattacked. This problem requires a separate investigation. It will be interesting to explore this problem further, since it is well known that brewer's and baker's yeasts possess the prototype system of the HMP-oxidative route, which also seems to be present in some moulds (Koffler, 1953).

Micro-organisms unable to decompose 2-ketogluconate. The remaining yeasts, moulds, bacteria and all the actinomycetes occur in this group, as summarized at the beginning of the section headed Results. The inability to demonstrate decomposition of 2-ketogluconate by these micro-organisms does not preclude the possible presence of an HMP-oxidative route.

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Properties of a Small Bacteriophage and the Action of some Compounds on it

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SUMMARY: Phage S13, a small phage active against a strain of *Escherichia coli*, had a latent period of 20 min. on this host. Intracellular phage multiplication was detected only toward the end of the latent period. Photo-reactivation of ultraviolet irradiated phage occurred, but the inactivated phage did not kill its host. More than 1600 compounds were tested for ability to prevent growth of phage S13 on *E. coli* at concentrations allowing growth of the bacterium, but only thirteen were active. Seven of these compounds were examined further; none affected free S13 phage or the adsorption of S13 phage to its host. The only compound investigated in detail, 4:4-bis-(2-dihydroglyoxalanyl) stilbene dihydrochloride, inhibited both early and late, but probably not intermediate, stages in the intracellular development of S13 phage. This was different from the action of this compound on T1 phage. No relationship was found between structure and action against phage, nor between compounds active against S13 phage and those active against larger phages.

As part of an investigation into virus chemotherapy, S13 phage of *Shigella paradysenteriae* (*S. flexneri*) Y6R was used as a representative of the smallest viruses. Elford & Andrewes (1932) by ultrafiltration estimated its diameter to be 8-12 m μ ., while Elford (1936) by a centrifugation method found it to be 15-17 m μ . i.e. it was of the same order of size as the viruses of poliomyelitis and foot and mouth disease. Because of its small size S13 phage might differ from larger phages in some of its properties. This paper reports studies on these properties, including the action of certain compounds on this phage, and a comparison of the activity of one of these compounds on S13 with its activity on T1 phage of *Escherichia coli*.

EXPERIMENTAL

S13 phage, and a strain of *Escherichia coli* against which S13 phage was active, were obtained through the courtesy of Dr C. H. Andrewes, F.R.S. In this paper the bacterium will be termed *E. coli* CHA.

Strains. Stock S13 phage was prepared by the method of Swanstrom & Adams (1951). Best results were obtained by concentrating a 24 hr. broth culture of *Escherichia coli* CHA tenfold by centrifugation and using the concentrate for sowing the plates. The lysate was filtered through a Gradocol membrane of porosity 0.9 μ . The final titre was 4×10^{10} particles/ml.

T1 phage was prepared by sowing a 2 hr. Lab-Lemco broth culture of *Escherichia coli* CHA with 10^4 particles of T1 phage previously obtained by lysis of *E. coli* B, incubating at 37° for 6 hr., then filtering through a Gradocol membrane of porosity 0.9 μ . The final titre was 3×10^9 particles/ml. T1 phage

prepared from *E. coli* B gave 100 times more plaques when plated on *E. coli* B than when plated on *E. coli* CHA. The phage obtained by lysis of *E. coli* CHA gave the same titre on both bacterial strains. The variation appears to be a phenotypic one similar to that described by Bertani & Weigle (1953).

Escherichia coli CHA grew (slowly) on a defined medium which contained lactic acid and ammonia as sole sources of carbon and nitrogen. It was sensitive to T1, T3 and T7 but resistant to T2, T4, T5 and T6 phages of *E. coli* B.

Media. Lab-Lemco broth was used as liquid medium and nutrient (tryptic digest) agar as solid medium.

Titration of phage. For one-step growth experiments: to 1 ml. phage suspension in a test tube, 1 ml. of a 24 hr. culture of *Escherichia coli* CHA and 3 ml. nutrient agar were added. After the contents had been mixed, the tubes were sloped and incubated at 37° for 4 hr. before reading. In all other experiments the mixture was poured on 15 ml. agar bases instead of sloping, and the plates incubated for 24 hr. at 37° before reading. The shorter period of incubation was necessary after sloping, as after 24 hr. plaques were too large for convenient numbers to be counted in tubes.

RESULTS

Stability of S13 phage. S13 phage was stable in Ringer's solution at 37° for at least 30 min. and at 5° for at least 6 months; at 60° the titre fell from 3×10^8 particles/ml. to 4×10^3 particles/ml. in 30 min. In M/15 phosphate + citrate buffer at 37°, S13 phage was stable at pH 8 for at least 1 hr.; at pH 5 the titre fell from 1×10^6 /ml. to 1×10^5 particles/ml. in 1 hr. pH values outside the range 5–8 were not investigated.

Latent period (Fig. 1). The method of Dickinson & Codd (1952) was used. To obtain good absorption a 2 hr. culture of *Escherichia coli* CHA was suspended in Ringer's solution, incubated at 37° for 5 min. only, and S13 phage added to give 2×10^8 host cells and 10^8 phage particles/ml. The mixture was incubated 10 min. before dilution into broth. The latent period was about 20 min.

No increase in the titre of S13 phage was found when KCN (final concentration 0.02 M; Rountree, 1951) was added at any time during the first half of the latent period (Fig. 1). Attempts to break open the bacterial cell at earlier times by the method of Rountree (1951) were unsuccessful.

Effect of ultraviolet radiation on S13 phage. S13 phage (4×10^9 particles/ml.) in broth was irradiated with a 15 W. 18 in. British Thomson-Houston germicidal lamp (which gave 99 % of its energy at a wavelength of 2537 Å.) at a distance of 37.5 cm. An exposure of 60 min. (the dose usually given) was required to decrease the titre one hundredfold. The irradiated phage had a significantly higher titre when absorbed on a 2 hr. culture of *Escherichia coli* CHA for 5 min. in daylight before counting than when counted immediately (Table 1). The effect increased with decreasing phage concentration over a fourfold range, so that it was not due to multiplicity reactivation. No increase occurred on dilution in absence of *E. coli* CHA. No work was done in absence of light but it seems reasonable to attribute the observed effect to photo-reactivation.

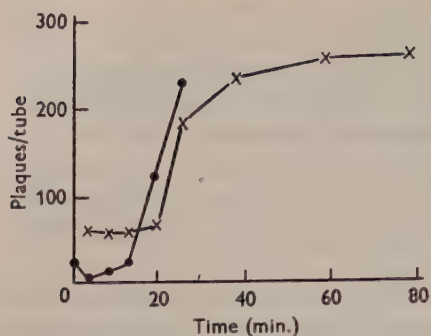


Fig. 1.

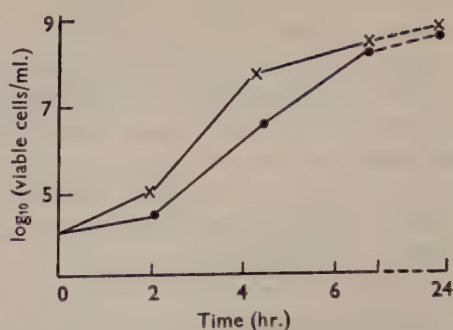


Fig. 2.

Fig. 1. Premature liberation of intracellular S13 phage by addition of 0.02 M-KCN at various times. \times — \times , latent period in absence of cyanide; \bullet — \bullet , phage yield when cyanide added at various times.

Fig. 2. The action of compound no. 347 (20 μ g./ml.) on the growth of *Escherichia coli* CHA. \times — \times , compound no. 347 absent; \bullet — \bullet , compound no. 347 present.

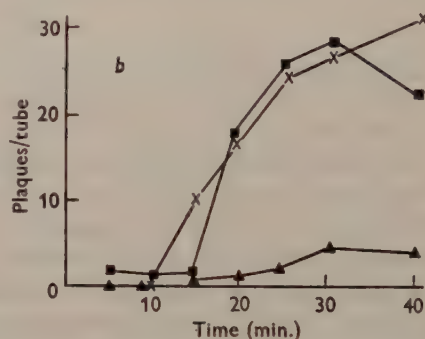
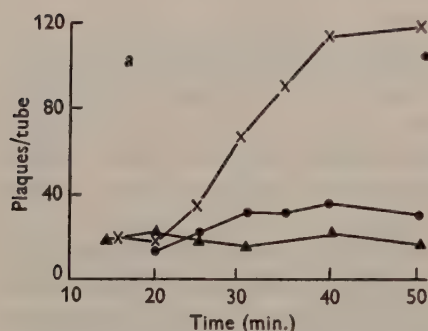


Fig. 3. The effect of compound no. 347 (20 μ g./ml.) on the latent periods on broth of (a) S13 phage, (b) T1 phage on *Escherichia coli* CHA. \times — \times , compound no. 347 absent; \blacktriangle — \blacktriangle , compound no. 347 present throughout; \blacksquare — \blacksquare , compound no. 347 added at 3 min.; \bullet — \bullet , compound no. 347 added at 20 min.

Table 1. Reactivation of ultraviolet-inactivated S13 phage in the presence of *Escherichia coli* CHA

Treatment	Host presence	Phage count (particles/ml.)
Unirradiated	—	1.6×10^9
Irradiated	—	1.6×10^7
Irradiated, diluted* 1:2	+	1.2×10^7
Irradiated, diluted 1:4	+	7.3×10^6
Irradiated, diluted 1:8	+	4.4×10^6
		Host count (no./ml.)
Host + broth	.	1.4×10^7
Host + unirradiated phage	.	8×10^4

* Dilutions were in broth. The first dilution was due to mixing equal volumes of phage and host. Phage and host were incubated together for 5 min. before counting.

In the course of this work it was noted that though infection of *Escherichia coli* CHA with normal S13 phage decreased the viable bacterial count, infection with ultraviolet (u.v.)-inactivated S13 phage failed to do so (Table 2). Studies on antiserum neutralization by u.v.-inactivated S13 phage failed to

Table 2. *Effect of ultraviolet-inactivated S13 and T1 phages on the viability of Escherichia coli CHA*

Equal volumes of host and phage were incubated together for 5 min. before host counts were made.

	S13 phage	T1 phage
	Phage count (particles/ml.)	
Before irradiation	4×10^9	2.5×10^9
After irradiation	5.6×10^8	5.8×10^8
	Host count (no./ml.)	
Host + broth	1.6×10^8	9.0×10^8
Host + unirradiated phage	3.6×10^7	1.4×10^4
Host + irradiated phage	1.7×10^8	1.5×10^4

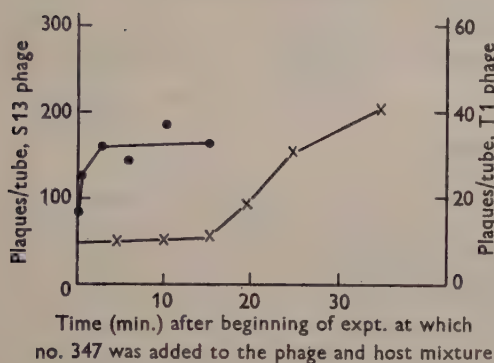


Fig. 4.

Fig. 4. Effect of compound no. 347 (20 $\mu\text{g./ml.}$) on the yields of S13 and T1 phages from *Escherichia coli* CHA when added at different times during their latent periods. ●—●, T1 phage; x—x, S13 phage. Tubes were 'sloped' at 60 min.

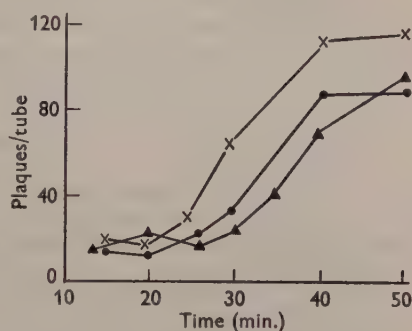


Fig. 5.

Fig. 5. The effect of diluting out compound no. 347 (20 $\mu\text{g./ml.}$) on the latent period of S13 phage on *Escherichia coli* CHA. x—x, compound no. 347 absent; ▲—▲, compound no. 347 initially present, diluted out at 15 min.; ●—●, compound no. 347 added at 5 min., diluted out at 15 min.

show whether it was adsorbed on *E. coli* CHA, but as reactivation occurred only in presence of *E. coli* CHA it appears probable that adsorption did take place. It was confirmed (Table 2) that u.v.-inactivated T1 phage killed *E. coli* CHA.

Action of various compounds on S13 phage

A preliminary test of each compound against S13 phage was made by a cup-plate diffusion method using a 1% (w/v) neutral aqueous solution or suspension of the compound in a single cup. Those compounds which gave an appreciable zone of phage inhibition were retested by the serial dilution method of Chantrill, Coulthard, Dickinson, Inkley, Morris & Pyle (1952), using five-fold

serial dilutions. Some of the compounds found active in this test were examined in greater detail by the method of Mills (1953).

Of more than 1600 compounds screened against S13 phage only thirteen were active by the serial dilution method (Table 3), though many showed appreciable activity in the cup-plate test. No reason was found for this

Table 3. *Compounds which prevented the growth of S13 phage on Escherichia coli CHA*

Ref. no.	Compound	Activity*	Action on absorption	Action on intracellular phage
347	4:4-bis-(2-dihydroglyoxalanyl) stilbene dihydrochloride, monohydrate	5.1/4.4p	0	+
348	2-hydroxy-4:4'-diamidinostilbene dihydrochloride, dihydrate	5.1/4.4p	Not tested	
690	phenyl guanidine	2.7/2.1	0	+
774	1:2-bis-(4'-benzamidinylphenoxy) ethane di-HCl	3.4/2.7	Not tested	
776	1:4-bis-(4'-benzamidinylphenoxy) butane dihydrochloride	3.4/2.7	0	+
1105	bis-(p-N-2-pyridylamidophenyl) ether	4.8/4.1	0	+
1202	di-(p-N-p'-methoxyphenylamidinophenyl) ether	4.8/4.1	Not tested	
1148	9-(p-aminoanilino) phenanthridine	5.1/4.4	0	+
1207	2:7-diamino-9-anilinophenanthridine	4.4/3.7	Not tested	
1367	2:7-bis-(2-dihydroglyoxalanyl) 9-phenylphenanthridine, trihydrochloride, dihydrate	5.1/4.4	0	+
2177	2:7-diamino-10-methyl-9-p-tolylphenanthridinium bromide	4.8/4.1	Not tested	
2202	2:7-diamino-9:10-dimethylphenanthridinium bromide	5.5/4.8	Not tested	
1475	2, 4, 5, 6-tetra-aminopyrimidine sulphate, monohydrate	3.4/2.7	0	+

* Activity is expressed as

- log (minimum concentration (g./ml.) preventing phage growth)
- log (minimum concentration (g./ml.) preventing host growth).

difference in response. No compound affected the titre of S13 phage at its active concentration, and none of seven compounds tested prevented adsorption of S13 phage on *Escherichia coli* CHA. All compounds tested except one, no. 347, 4:4-bis-(2-dihydroglyoxalanyl) stilbene dihydrochloride, decreased host growth too much at the active concentration for more detailed work to be of value.

Action of compound 347

In a one-step growth experiment compound 347 prevented phage multiplication at 20 µg./ml. and caused little phage loss (Fig. 3a). This concentration was used for the rest of the work reported here. Compound 347 did not diminish the titre of free S13 phage (Table 4), nor did it prevent a burst when present during absorption if, after absorption, it was diluted well below the limit of activity (Table 5). It slightly retarded the growth of *Escherichia coli* CHA as measured by plate colony counts (Fig. 2).

Action on intracellular S13 phage. Compound 347 was added at various times

during the latent period to samples of the phage + host system, and left till the burst had ended. There was no phage multiplication when compound 347 was added during the first 15 min. When added after 15 min., phage yield increased the more the later compound 347 was added (Fig. 4). When added at 20 min. compound 347 did not increase the latent period (Fig. 3a).

Table 4. *Action of compound 347 on free S13 and T1 phages in broth*

Time (hr.)	Concentration of compound 347			
	0		20 μ g./ml.	
	S13 phage (phage count $\times 10^{-4}$)		T1 phage (phage count 1.25×10^{-5})	
0	47, 40		22, 17	
1	28, 22		23, 21	

Table 5. *Action of compound 347 on the adsorption of S13 and T1 phages on Escherichia coli CHA*

Concentration of compound 347 was 20 μ g./ml.

Presence (+) or absence (-) of compound 347		S13 phage, plaques/tube at		T1 phage*, plaques observed	
				Diluted 1/10 at 7 min.	Diluted 1/100 at 60 min.
		10 min.	60 min.		
-	-	30, 29, 38	133, 183, 158	26, 23, 23	80, 79, 93
-	+	28, 25, 15	15, 6, 6	27, 26, 29	39, 36, 29
+	-	29, 25, 31	135, 155, 180	29, 23, 22	71, 77, 84
+	+	23, 19, 27	15, 6, 15	23, 28, 29	3, 5, 2

* In the experiment on T1 phage, the phage was diluted ten-fold immediately before counting at 7 min. and 100-fold immediately before counting at 60 min. The figures given are the counts observed.

Effect of diluting out. When compound 347 was present initially and was diluted out at 15 min., the latent period was lengthened by about 10 min. (Fig. 5). The results of this and the preceding experiments suggested that compound 347 was acting at the beginning and at the end of the latent period. To determine whether it was also acting at intermediate stages it was added at 5 min., diluted out at 15 min., and the latent period measured. The results (Fig. 5) were equivocal but suggested that any action it had on intermediate stages could only be slight.

Effect of the active compounds on T1 coliphage

For comparison with the results reported above, some experiments were made on T1 coliphage grown on *Escherichia coli* CHA, using the same methods of testing. Eleven of the thirteen compounds active against S13 phage were tested by the serial dilution method against T1 phage; only three were active, including compound 347, and a few experiments on its mode of action were

made. It prevented multiplication of T1 phage on *E. coli* CHA at 0.3 $\mu\text{g./ml.}$, compared with 8 $\mu\text{g./ml.}$ for S13 phage. At 20 $\mu\text{g./ml.}$ it had no action on free T1 phage (Table 5), nor on the absorption of T1 phage on to *E. coli* CHA. It had little effect on the yield of T1 phage when added at 20 $\mu\text{g./ml.}$ at 3 min. or more after the start of the latent period (Figs. 3*b* and 4).

DISCUSSION

The only unusual property observed of the S13 phage + *Escherichia coli* CHA system was the failure of S13 phage after inactivation by ultraviolet irradiation to kill its host. In this it differed from T2 (Dulbecco, 1952) and T1 coliphages. On the other hand, S13 phage, in its response to the compounds tested for inhibitory activity against it, differed considerably from certain larger phages. Thus proflavine was inactive against S13 phage though active against T2 phage (Foster, 1948), Pb phage of *Pseudomonas aeruginosa* (Dickinson, 1948) and staphylococcus phage K (Hotchin, 1951): several phenanthridines active against Pb phage (Dickinson, Chantrill, Inkley & Thompson, 1953) were inactive against S13 phage, while two (nos. 1148 and 1207) were active against S13 phage but not Pb phage. Of the eleven compounds active against S13 phage and tested against T1 phage, only three (nos. 347, 1367 and 2202) were active against the latter. While there was no relationship between activity against S13 phage and against larger phages, equally it was clear from consideration of the active compounds (Table 3) and certain inactive ones (not listed here) that there was no relationship between the structure of a compound and its activity against S13 phage.

The only compound (no. 347) whose action on S13 phage was studied in detail had previously been reported to be active against Pb phage of *Pseudomonas aeruginosa* (Dickinson & Codd, 1952). Its action on T1 phage resembled that of the phenanthridine no. 1367 on Pb phage (Mills, 1953; Table 3). Its action on S13 phage appeared to combine such an action with one similar to that of proflavine on T2 phage (Foster, 1948).

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Studies in the Differentiation between Human and Animal Pollution by means of Faecal Streptococci

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With a Note on the Standardization of the Janus Green Milk Test

SUMMARY: The isolation of faecal streptococci from the excreta of man, cattle and sheep was investigated. The methods compared were (a) heat, (b) tetrathionate, (c) tellurite, (d) thallium salts. The tellurite method proved highly efficient, isolating 97 % of strains from human, cow and sheep faeces. A survey of the properties of strains isolated was made. Differential tests based on (a) heat resistance and (b) reducing properties were devised; these divided the streptococci isolated by the tellurite method into groups, some of which were characteristic of the source. The information available seems capable of distinguishing many strains obtained from man from those derived from cows or sheep, and it is suggested it may be of use in tracing sources of pollution in water, milk and other foodstuffs.

No satisfactory method exists at present for distinguishing faecal pollution by human sources from that by other animal sources. The chief organism used in proving faecal pollution, namely *Escherichia coli* type I, may arise from either source (see Rep. Minist. Hlth, Lond. 1940). Faecal streptococci have also been advocated as confirmatory evidence of pollution, but the significance of different types has been little investigated. The nature of the types isolated from faecal material depends to some extent on the method of isolation. The strains isolated by the two methods proposed in the Ministry report, namely the heating method and the tellurite method, are often different. Other methods of isolation are also possible. Cooper, Baker, Elliot & Wood (1942) reported the isolation by tetrathionate media, and Cooper & Linton (1947) showed the resistance of these organisms to thallium acetate. The use of sodium azide was investigated by Allen, Pierce & Smith (1953).

The present paper describes the results of experiments which compared these various methods (except the use of azide), and a more extended investigation of the streptococci obtained from human, cow and sheep faeces by the tellurite method. As a result it appears that a high percentage of strains have properties which enable their source to be determined with considerable certainty. The faecal streptococci, therefore, in contrast to *Escherichia coli* may prove of value in tracing pollutions in water, milk and other foodstuffs.

The isolation of streptococci by tetrathionate broth and by heat (60° for $\frac{1}{2}$ hr.) was compared by Cooper, Baker, Elliot & Wood (1942), and though equally successful from human faeces, showed that heat destroyed many strains in cattle excreta. That investigation, and a further one in 1950, revealed that three-quarters of the streptococcal strains isolated from cattle and sheep produced green-yellow discoloration on heated blood agar. Less than one-

quarter of the human strains did this; for the media used see Cooper, Mayr-Harting & McLachlan (1950). However, variability of the results on repeated subculture and with changes in the media led us in this investigation to study reducing properties instead of the production of peroxides.

SURVEY OF ISOLATION METHODS

Thirty specimens of faeces from human, cow and sheep sources were concentrated in three different media, namely:

(a) *Tetrathionate broth* as used for the isolation of *Salmonella paratyphi* B from faeces (Cooper, Wood, Elliot, Caswell & Small, 1942).

(b) *Potassium tellurite medium*: a modification of the medium of Harold (1936) for isolation of streptococci from water was used; potassium tellurite, final concentration 1/5000 in 0.5 % lactose, 0.5 % peptone water buffered at pH 7.6 with 0.5 % potassium phosphate.

(c) *Thallium acetate medium* which contained 0.5 % peptone, 0.5 % lactose, 0.5 % anhydrous dipotassium phosphate and thallium acetate to make final concentration 1/2000 (Cooper & Linton, 1947).

Method. A loopful of excreta was emulsified in 10 ml. sterile water; filtered through four layers of muslin, the filtrate mixed and with a Pasteur pipette 10 drops inoculated into tubes of each of the three media. Plate cultures were made after 24 hr. on to MacConkey agar (0.5 % sodium taurocholate) and incubated for a further 24 hr. Streptococcal colonies were picked and subcultured on 10 % heated blood agar until pure. A well-defined colony was distributed in Bactopeptone broth and then incubated at 37° for 16 hr. The resulting growth was examined microscopically and subjected to a series of biochemical tests to study the following properties:

Haemolysis. Shattock's modification (1949) of Brown's technique.

Gelatin liquefaction. Stab cultures observed over 14 days at room temperature.

Action in litmus milk at 37°. The term 'strong litmus reduction' was applied to cases where the dye was totally reduced in the depths of the tube in 24 hr. with or without colour change at the surface. The medium (5 ml.) was in screw-cap bottles (1 oz. McCartney), incubated with the stopper tight and not shaken.

Survival at 60° for 30 min. A sample (0.5 ml.) of culture in Bactopeptone broth was transferred into a Widal tube (65 × 9 mm.) and placed in a water-bath at 60° with the level of culture well below the water and heated for 30 min. The tubes were then transferred to another water-bath at 37° and kept for 2 hr. Subcultures were then made on 10 % heated blood agar and plates incubated for 48 hr. and examined for growth.

Growth at pH 9.6. Method of Shattock & Hirsch (1947).

Inhibition by 6.5 % sodium chloride. Method of Abd-el-Malek & Gibson (1948).

Inhibition by 0.1 % methylene blue. A loopful of culture was inoculated in 5 ml. sterile skimmed milk containing 0.1 % methylene blue and incubated in a water-bath at 37°. The results were read after 24 hr.

Growth on 40 % ox bile medium. Abd-el-Malek & Gibson (1948).

Starch hydrolysis. Poured plate cultures made by using 1 ml. culture and melted 0.2 % starch agar cooled to 45°. Hydrolysis tested with iodine solution after 5 days at 37°.

Sodium hippurate hydrolysis. Ayers & Rupp (1922).

Sugar fermentation reactions were observed in peptone water to which was added a sterile solution of 0.5 % sucrose, lactose, raffinose, inulin, mannitol, or 1 % glycerol, and phenol red.

RESULTS

Isolation methods

By using the three selective media 195 strains of streptococci were isolated from the ninety specimens of faeces. The relative number of strains recovered by each medium in accordance with its value as a method of isolation is shown in Table 1. *Tetrathionate broth*, though often successful with human faeces, failed rather significantly in concentrating streptococci from sheep and bovine faeces. *Proteus vulgaris*, micrococci and yeasts were usually prevalent.

Table 1. *Incidence of streptococci isolated by three different methods*

Faeces		Method of isolation						Total strains isolated
		Tetrathionate		Tellurite		Thallium acetate		
		No. of strains	% of success	No. of strains	% of success	No. of strains	% of success	
Human	30	18	60	30	100	24	80	72
Bovine	30	1	3	28	93	28	93	57
Sheep	30	7	23	29	96	30	100	66
Total	90	26	29	87	96.7	82	91.1	195

The strains isolated by each method usually differ in properties and have therefore been regarded as distinct, though originating from the same specimen.

Potassium tellurite medium proved to be highly selective in concentrating streptococci from human and animal faeces (97 % of specimens). The advantage of a concentration of 1/5000 is that it helps to diminish *Proteus vulgaris* and fungi which are often predominant in animal faeces. Fleming (1932) discovered that some proteus strains resist 1 : 8000 potassium tellurite and that 1 : 5000 did not affect faecal streptococci. Additional experiments with solid media revealed that faecal streptococci from man, cow and sheep multiplied on nutrient agar or (10 %) heated blood agar containing 0.2 % potassium tellurite.

Thallium acetate medium may be considered equal to tellurite medium in securing heavy pure growth of streptococci from animal faeces but it failed in a considerable number of cases with human faeces.

Strains isolated

By using the three methods of isolation it was possible to obtain at least one streptococcus strain from every specimen of faeces. Usually strains with different characters were recovered from the same specimen when treated with different methods. Strains which showed all the classical characteristics of named species have been so designated. In an endeavour to classify the other streptococci into convenient groups the first eight characters presented in Table 2 were taken as the basis for differentiation. According to this method, 40 % of human-derived streptococci were grouped as *Streptococcus faecalis*, a specific name given by Andrewes & Horder (1906) and firmly established by Dible (1921) and Sherman (1938). Typical *S. faecalis*, as described in Table 2, did not exist among streptococci derived from cow and sheep faeces. *S. durans* was also only recovered from human faeces and represented 4 % of human strains. *S. faecalis* var. *liquefaciens* and *S. faecalis* var. *zymogenes* accounted for about 29 % of the human-derived streptococci. The liquefaciens variety represented a group of 14 % of bovine and 18 % of sheep strains. The zymogenes variety was not encountered among the bovine organisms but formed 6 % of streptococci from sheep. Many investigators have recovered the liquefaciens and zymogenes variants from human faeces, milk, ice-cream, dried milk and cheese, but there seems no evidence from the available literature that they have been noticed before in bovine or sheep faeces.

Human, bovine and sheep faeces share in common several groups of streptococci which differ from the typical *Streptococcus faecalis* in one or more essential criteria and usually other secondary ones. Since the object of the present work is the subdivision of streptococci from various sources into groups convenient for differential purposes, the name 'Atypical faecalis' designated I, II, III, IV and V was applied to those strains which differed from the typical faecalis in one, two or three standard characters. These undoubtedly would include most of the unclassified and the so-called *S. faecalis* 'variants' and 'enterococcus-like' organisms recorded by several investigators.

Only one strain of *Streptococcus bovis* was encountered among the human-derived collection. Unlike the other *S. bovis* strains this one failed to ferment starch. Orla-Jensen (1942) observed that human strains of *S. bovis* did not hydrolyse starch. *S. bovis* represented 28 % of streptococci recovered from cow dung; none was found in sheep dung. The presence of *S. bovis* in small numbers in the human intestine had been recognized by many investigators; Sharpe (1948) encountered thirty-seven *S. bovis* strains out of 340 streptococcal strains recovered from the faeces of human infants. This high incidence may be attributed to the common presence of this organism in cow's milk which may be incompletely sterilized for infant feeding. The recovery of *S. bovis* in only 28 % of bovine-derived strains is a result different from that of Ayers & Mudge (1923) who considered *S. bovis* as the predominating streptococcus in bovine intestine. This difference in findings may be due to the methods of isolation used or to the different criteria for identification. None

Table 2. The physiological characters of streptococci from human, bovine and sheep faeces

Origin of streptococci	Classification	No. of strains	No. of strains re-covered by each method	Haemolysis	Gelatin	Strong litmus milk reduction	Mannitol	Sucrose	Raffinose	Survival at 60° for 30 min.	Growth at pH 9.6	Growth in 6.5% NaCl	Starch hydrolysis	Growth in 0.1% M.B. milk	Sodium hippurate hydrolysis	Inulin	Glycerol	Sorbitol
Human faeces	<i>S. faecalis</i> (typical)	29	a. 5 b. 15 c. 9	-	-	+	A.	A.	-	+	+	+	-	+	+	-	A.	A.
	<i>S. faecalis</i> var. <i>liquefaciens</i>	11	a. 3 b. 5 c. 3	-	+	+	A.	A.	-7 +4	+	+	+	-	+	+8 -3	-	A.	A.
	<i>S. faecalis</i> var. <i>zymogenes</i>	10	a. 2 b. 3 c. 5	+	-7 +3	+	A.	A.	-	+	+	+	-	+8 -2	+9 -1	-	A.	A.
	<i>S. durans</i>	3	b. 1 c. 2	+	-	-	-	-2 +1	-	+	-	+2 -1	-	-	-2 +1	-	-	-
	<i>S. bovis</i>	1	b. 6	-	-	-	A.	A.	A.	-	-	+	-	-	+9 -2	A.	-	A.
	Atypical faecalis I.	11	b. 4 c. 1 b. 1 c. 4	-	-	-	A.	A.	-	+	± V.sl.	+	-	+w.	-2	-	-	A.
	Atypical faecalis II.	7	a. 2 b. 1 c. 4	-	-	-	A.	A.	-	+	± V.sl.	+	-	-	+w.	-	-	A.
	<i>S. bovis</i>	16	b. 10 c. 6	-	-	-	-	+14 -2	A.	-	-	-	+	-	-	A.	-	-
	<i>S. faecalis</i> var. <i>liquefaciens</i>	8	b. 5 c. 3	-	+	+	A.	A.	-	+	+	+	-	+	+4 -4	-	A.	A.
	Atypical faecalis I.	12	a. 1 b. 6 c. 5	-	-	-	A.	A.	-	-	+	+	-	+7w. -5	+4	-	-7 +5	-7 +5
Bovine faeces	Atypical faecalis III.	10	b. 4 c. 6	-	-	-	A.	A.	A.	-	+	+	-	-7 +3	-	-	+6 -4	+8 -2
	Atypical faecalis IV.	8	b. 2 c. 6	-	-	-	A.	A.	A.	+	± V.sl.	+5 -3	+	+4w. -4	+3 +w.	-	-	-
	Atypical faecalis V.	3	b. 1 c. 2	-	-	-	-	A.	-	+	+	+	-	-	-	-	-	-
	<i>S. faecalis</i> var. <i>liquefaciens</i>	12	a. 1 b. 6 c. 5	-	+	+	A.	+10 -2	-	+	+	+	-	+	+7 -2	-	+10 -2	+10 -2
	<i>S. faecalis</i> var. <i>zymogenes</i>	4	b. 2 c. 2	+	+2 -2	+	A.	A.	+2 -2	+	+	+	-3 +1x	+w.	+2 -2	+2 -2	A.	A.
	Atypical faecalis II.	26	a. 4 b. 9 c. 13	-	-	-	A.	A.	-	+	+14 +12 V.sl.	+	-	-	-	-	-22 +4w.A.	+19 -7
	Atypical faecalis III.	15	b. 9 c. 6	-	-	-	A.	A.	A.	-	+	+	-	-	-	-	-	-
	Atypical faecalis V.	9	a. 2 b. 3 c. 4	-	-	-	-	A.	-	+	+	+	-	+	-5 +4w.	-	-	-
				-	-	-	-	A.	-	+	+	+	-	+	-	-	-	-
				-	-	-	-	A.	-	+	+	+	-	+	-	-	-	-
Sheep faeces				-	-	-	-	A.	-	+	+	+	-	+	-	-	-	-
				-	-	-	-	A.	-	+	+	+	-	+	-	-	-	-

of our *S. bovis* strains was capable of surviving a temperature of 60° for 30 min. This is contrary to Sherman's findings (1937) and agrees with observations made by Shattock & Mattick (1943). The discrepancy may be due to differing amounts of peroxide formed in varying media, as a lethal factor additional to the temperature.

SURVEY OF DIFFERENTIAL TESTS FOR INDICATING SOURCE

The tests used to distinguish species within this group have increased in number and variety in recent years, and this has resulted in a multiplicity of recognizable types. Which of these types are worthy of being called species, or indeed, which of the tests employed should be regarded as diagnostic and which as only showing minor variants is still disputed. We are not here primarily concerned with classification but with the value of some of these tests as an indication of the sources of the strains concerned.

Choice of method of isolation

The method of isolation itself determines some of the characteristics of the strains isolated. The use of 1/5000 potassium tellurite followed by subculture to MacConkey agar (0.5 % sodium taurocholate) isolated a high proportion (97 %) of the bile-resistant enterococci from faeces of man, cow and sheep. This work requires extension to other sources of pollution such as birds, pigs, dogs, cats, rabbits, rats, etc.; nevertheless, the strains thus isolated probably represent the predominant organisms likely to gain access to water or milk supplies. Isolations by this method were therefore made from 350 specimens of faeces (130 human, 110 cow, 110 sheep). Bactopeptone broth cultures (16 hr., 37°) of the purified strains isolated were prepared for examination by different tests (343 strains).

Choice of differential tests

Earlier work had suggested the value of heat-resistance tests and of tests for oxidizing or reducing ability. The difference between human and animal strains was of a quantitative nature and best demonstrated on freshly isolated strains. With a view to avoiding, if possible, prolonged quantitative measurements it was decided to utilize a dye which indicated different degrees of reduction, and Janus Green in milk was therefore compared with the previously used litmus milk. The heat test was made a little more severe (63° for 30 min.), and the recovery of strains was tested not only in a rich medium (10 % heated blood agar), but also on McLeod's tellurite medium (Anderson, Happold, McLeod & Thompson, 1931).

Heat-resistance tests. A sample (0.5 ml.) of culture was transferred into a Widal tube (65 × 9 mm.); the tube placed in a water-bath at 63° with the level of the culture well below the water and heated for 30 min. The tube was then transferred to another water-bath at 37° and kept for 2 hr. With a 4 mm. diameter loop one loopful was subcultured: (i) to a (10 %) heated blood agar

and the plate incubated for 48 hr. after which it was examined for growth; (ii) to McLeod's tellurite medium and the plate incubated at 37° for 48 hr. and examined for growth.

Janus Green milk test. Janus green B (dimethylsafranin azodimethylaniline; a Gurr preparation; batch no. 1041, see p. 189) was dissolved in sterile glass-distilled water to form a 1 % solution. The dye solution was heated in a water-bath at 80–100° for 15 min. and left to cool. It was then added to sterile separated milk to bring the final concentration to 1/10,000 and the medium distributed in 5 ml. amounts in screw-capped bottles. One loopful (4 mm. diameter) of inoculum from the broth culture was transferred to a screw-capped bottle of medium and incubated along with a non-inoculated bottle of medium as control in a water-bath at 37°. Readings were made after 16 hr. to record the rate of reduction of the dye in the milk culture.

Litmus milk reduction test. One loopful (4 mm. diameter) of broth culture was put in 1 % litmus milk and the bottle incubated in a water-bath at 37° for 16 hr. after which the changes and rate of reduction of litmus in the milk culture were recorded. These technical procedures were conducted on adequate numbers of human, cow and sheep-derived strains in parallel, so that the conditions under which these strains were tested may be considered comparable.

RESULTS

The tellurite method isolated streptococci from 127 (97·6 %) of 130 samples of human faeces; from 106 of 110 samples (96·3 %) of cow faeces and from 110 of 110 (100 %) samples of sheep faeces, which when examined by the above procedure revealed the findings stated in Table 3. It was found that when Janus Green B underwent reduction in a milk culture, a series of changes in colour occurred in the following order: blue → mauve → violet → dirty red → wine red (quinonoid) → rosy pink → pale pink → colourless. The Janus Green milk test was considered positive when the colour of the dye reached after 16 hr. at 37° was the quinonoid red (semi-reduced form) or any shade following it towards the leucoform of the dye. The rosy pink colour, however, was that most often observed in positive Janus Green milk tests. On the other hand, a negative result was taken as indicated by any culture in which the colour lay between the blue and the dirty red shades. To avoid confusion between 'dirty red' and 'quinonoid red', the cultures were shaken before examination, thus allowing atmospheric oxygen to restore the violet shade to cultures which had not been reduced to the quinonoid red form.

The Janus Green milk test revealed 85 % positive results among strains of human origin, and only 19·4 % among those of animal origin. The results obtained with the litmus milk test showed that 72·4 % of human-derived strains and 46·2 % of the animal-derived ones were capable of reducing the dye to the colourless form during the same period of incubation.

Janus Green has shown itself to be a better indicator than litmus of the relative degree of reduction of streptococcal milk cultures. Unlike litmus, Janus Green does not change colour so readily with pH changes of the medium

which makes it a more suitable indicator for the present purpose of indicating degrees of reduction. In comparing the results of the two tests on the same strains under the same conditions, not only did the Janus Green test show superiority in detecting a higher percentage of strong-reducing strains among the human-derived cultures, but it also gave less than half the percentage of reducing strains among the animal-derived cultures than did the litmus test. About 50 % of cultures which did not reduce Janus Green within 16 hr. did so on incubation for a further 24 hr. This suggests that differences among excremental streptococci in their rate of Janus Green reduction are mainly due to differences in ability to reduce the dye rather than differences in their susceptibility to the antiseptic effect of the dye.

Table 3. *Incidence of positive and negative biochemical tests shown by streptococci of different origin*

Origin	Total no. of strains	Heat-resistance test (63°, 30 min.)	Heat and tellurite resistance test	Janus Green milk test	Litmus milk test
Human	127	+ 115 (90.5 %) - 12 (9.5 %)	+ 97 (76.4 %) - 30 (23.6 %)	+ 108 (85 %) - 19 (15 %)	+ 92 (72.4 %) - 35 (27.6 %)
Cow	106	+ 22 (20.7 %) - 84 (79.3 %)	+ 0 (0 %) - 106 (100 %)	+ 21 (19.8 %) - 85 (80.2 %)	+ 44 (41.5 %) - 62 (58.5 %)
Sheep	110	+ 35 (31.8 %) - 75 (68.2 %)	+ 0 (0 %) - 110 (100 %)	+ 21 (19 %) - 89 (81 %)	+ 56 (50.9 %) - 54 (49.1 %)

The heat-resistance test revealed that 90.5 % of the human and 26.3 % of the animal strains were thermotolerant. Heating at 63° was the temperature of choice. It gave a better differential test than that at 60°. It may be worth recording that some human strains were found to resist heating at 63° for 1 hr. and some for 90 min.

Faecal streptococci, when subjected to the heat-test fell into three categories: (a) strains killed by heat; (b) strains which survived the heat test but were subsequently not able to survive on the inhibitory medium; (c) strains which survived the two processes. Most of the heat-resistant strains from human faeces (84.3 %) qualified as category (c), i.e. survived both processes; none of the animal strains which survived the heat test belonged to this category.

The inhibition of growth of heat-treated cells on a 0.04 % potassium tellurite medium seems to be due to a weakness in the resistance of these cells acquired as a result of heat treatment, rather than to a bactericidal action of potassium tellurite. These same strains when unheated produced profuse black growth on McLeod's medium. That 0.04 % potassium tellurite is an important factor in the disinfection process was shown by the observation that a medium containing 0.02 % of that chemical generally supported the growth of heat-treated cells.

Significance of differential tests

When the strains examined are classified according to the two heat-resistance tests and the Janus Green reduction test into the six possible groups (Table 4), then the source of strains falling into some of these groups seems to be indicated

clearly. Groups I and II were derived entirely from human sources; other animal sources accounted for 98.5 % of group III and 91 % of group IV. Strains with the characters of groups V and VI were less characteristic of a particular source.

Table 4. *Biochemical grouping of faecal streptococci*

Group	Janus Green B milk test	Heat- resistance test	Heat and tellurite resistance test	Incidence							Total 343
				Human- derived strains (127)		Cow-derived strains (106)		Sheep-derived strains (110)			
				% of group		% of group		% of group			
				No.	% of group	No.	% of group	No.	% of group		
I	+	+	+	84	100	Nil	0	Nil	0	84	
II	—	+	+	13	100	Nil	0	Nil	0	13	
III	—	—	—	2	1.5	68	50.4	65	48.1	135	
IV	—	+	—	4	8.8	17	37.6	24	53.3	45	
V	+	+	—	14	46.7	5	16.7	11	36.7	30	
VI	+	—	—	10	27.8	16	44.4	10	27.8	36	

Conclusions

Whether strains with these characteristics will be as diagnostic of their source when a wider field is surveyed, remains to be seen. Our results suggest that with carefully devised tests it should be possible to distinguish human pollution from animal pollution by means of the faecal streptococci. The application of such tests after the tellurite method of isolation should be further investigated, and also compared with results by other methods of isolation such as the glucose + yeast-extract + sodium azide method advocated by Allen *et al.* (1953). The final identification of a strain by the use of most of the tests used in Table 2 will sometimes give more conclusive results. The characterization of a strain as typical *Streptococcus faecalis* seems to indicate a human origin. A starch-positive *S. bovis* points, on the other hand, very definitely to an animal origin.

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Note on the Standardization of the Janus Green Milk Test

Because the dye Janus Green B (dimethylsafranin azodimethylaniline: a Gurr preparation, batch no. 1041) was manufactured mainly for staining purposes and no actual account was recorded of its impurities or ash content, it was found necessary to standardize fresh batches of the dye against this original preparation. The dye preparations examined were: (1) Janus Green B (Gurr preparation, batch no. 351); (2) Janus Green (British Drug Houses Ltd. preparation, batch no. 183914); (3) Original batch, namely Janus Green B (Gurr preparation batch no. 1041). The depth of the blue colour produced by these dye preparations in sterile milk showed that the B.D.H. batch (no. 183914) and the original Gurr batch (no. 1041) both produced a deep blue colour in final concentrations of 1/10,000; but the other Gurr batch (no. 351) only showed a faint bluish tint in a final concentration reaching 1/5000.

Preparation of Janus Green milk. A 1% solution was prepared from each dye batch in sterile glass-distilled water, heated in a water-bath at 80–100° for 15 min. and then cooled. Bulk skimmed milk sterilized by steaming, was divided into three separate portions which were treated as follows:

The first portion. From batch 1041 (Gurr) the dye was added to the milk to produce a 1/10,000 concentration, and then distributed in 5 ml. amounts in

screw-capped bottles. This batch formed the original Janus Green B milk to be considered as the basis of standardization.

The second portion. The milk was divided into four parts. From the 1% solution of the batch no. 183914 (B.D.H.) the dye was added, in different proportions, to bring the final concentrations in these milks to 1/12,000, 1/10,000, 1/8000, 1/5000, respectively. The milk and dye was then distributed into screw-capped bottles in 5 ml. amounts and labelled.

The third portion was treated as the second portion except that dye batch no. 351 (Gurr) was used instead of the B.D.H. batch.

Test cultures. Sixty cultures of excremental streptococci from the collection isolated by the tellurite method (20 human, 20 cow and 20 sheep) were sub-cultured in Bactopeptone broth and incubated at 37° for 16 hr.

Method. One loop (4 mm. diam.) inoculation was made from each of the 16 hr. Bactopeptone broth cultures into bottles of Janus Green milk from the three different batches. The milks were then incubated in a water-bath at 37° with uninoculated controls. Readings were made after 16 hr.

Results. The original Janus Green B milk test (containing 1/10,000 Janus Green B batch no. 1041, Gurr) revealed that twenty-eight cultures (16 human, 12 animal) reduced the dye to the quinonoid red or pale pink form within 16 hr. at 37°, i.e. gave a positive test. In the remaining thirty-two cultures the dye remained oxidized (blue) or was only partially reduced (mauve, violet, dirty red), i.e. gave a negative test.

With the other dye+milk preparations, only the 1/8000 with batch no. 183914 (B.D.H.) milk test reproduced the same results as of the original test.

The 1/10,000 (B.D.H.) milk test, on the other hand, gave nine 'false-positive' results, while the 1/5000 concentration of the same batch showed an over-poising action in eleven cultures (i.e. false-negative results). Janus Green B (batch no. 351, Gurr) in the various concentrations used in milks, was reduced by all the strains involved in this investigation, either to the pale pink or the leucocolour.

From these results it may be assumed that in order to maintain its differential value between excretal streptococci, the Janus Green milk test should either be prepared in 1/10,000 final concentration from the 'Gurr preparation batch no. 1041' or in 1/8000 final concentration from the 'B.D.H. preparation batch no. 183914'. Every batch should be standardized, and not all are suitable. A small stock of B.D.H. preparation no. 183914 has been reserved to standardize new batches of Janus Green.

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Haemin and Isoniazid Resistance of *Mycobacterium tuberculosis*

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SUMMARY: In confirmation of Fisher's (1952, 1954*a, b*) results, haemin was found to act as an apparent growth factor for isoniazid-resistant strains of tubercle bacilli and also powerfully to antagonize the action of isoniazid on drug-sensitive organisms. In extension of Fisher's work, these effects of haemin were investigated with drug-sensitive and resistant strains of *Mycobacterium tuberculosis* H37Rv and B.C.G. The effect of Tween 80 and albumin was also examined. In Fisher's medium when the nitrogen source was changed isoniazid-resistant strains no longer appeared to be haemin-dependent. Middlebrook's observations that isoniazid-resistant tubercle bacilli are deficient in catalase (Middlebrook, 1954) and that catalase enables resistant organisms to grow in a deficient medium (Middlebrook, personal communication) were confirmed. Since the dependence of isoniazid-resistant organisms on haemin or catalase seems itself to be dependent on the presence of asparagine in the medium, it is suggested that haemin has a catalase or peroxidase-like role, and that in the presence of asparagine isoniazid-sensitive organisms can remove peroxide, whereas isoniazid-resistant organisms cannot.

Several lines of work have suggested that the action of isoniazid on mycobacteria is in some way related to their porphyrin-containing enzymes. Fisher (1952) found that certain isoniazid-resistant strains of *Mycobacterium tuberculosis* were unable to grow in a simple salts + asparagine medium which gave good growth of the parent isoniazid-sensitive strain. The resistant strains grew when serum or bovine albumin fraction V was added. Fisher later reported that the factor involved appeared to be haemin, and further, that haemin very powerfully antagonized the action of isoniazid on sensitive organisms (Fisher, 1954*a, b*). Gray (1953) suggested that the unique sensitivity of mycobacteria to isoniazid was due to an interaction between this compound and the relatively small quantity of haem catalysts known to be present in some members of this group of organisms (Finlayson & Edson, 1949). Middlebrook (1954) reported that whereas isoniazid-sensitive tubercle bacilli possessed catalase, this enzyme was absent from isoniazid-resistant variants of the strains tested.

Long chain fatty acids such as oleic acid have been shown to exert a dual effect on tubercle bacilli (Dubos, 1946). Free oleic acid may be inhibitory in minute amounts, but water-dispersible esters such as Tween 80 (the polyoxyethylene ester of sorbitan mono-oleate) may stimulate growth, especially when freedom from toxic amounts of free oleic acid is ensured by the addition of albumin (Dubos & Davis, 1946).

Some preliminary experiments suggested that an isoniazid-resistant strain of *Mycobacterium tuberculosis* H37Rv was much more sensitive to the inhibitory effect of Tween 80 than the corresponding isoniazid-sensitive strain whose growth seemed to be stimulated by it. Since it was known (Wright &

van Alstyne, 1931) that haemin catalyses the oxidation of 'oleic' acid by gaseous oxygen, it seemed possible that the growth-promoting effect of haemin described by Fisher might really be due to its efficiency in removing small amounts of oleic acid or other toxic substances. It was decided therefore to investigate the effect of haemin alone and in the presence of Tween 80 and albumin on the growth and isoniazid sensitivity of *M. tuberculosis*. The experiments here described have fully verified Fisher's main results, but further work has led to the conclusion that haemin is not a genuine growth factor in the sense of supplying a nutritional need. Haemin seems rather to act in a catalase-like role by destroying H_2O_2 or organic peroxides; the present paper gives the evidence for this and puts forward a working hypothesis of isoniazid action.

MATERIALS AND METHODS

The basal medium, prepared as described by Fisher (1954*b*), contained asparagine, phosphates, citrate, magnesium sulphate and glycerol, and was adjusted to pH 6.8. Additions or modifications were made as described in the text. A 9% (w/v) solution of bovine albumin fraction V (Armour) in water was Seitz-filtered and diluted 1/25. Tween 80 (10%, w/v) was diluted 1/200. Haemin (Light's Chemicals Ltd., Colnbrook, Bucks) was dissolved in 0.005 N-NaOH and catalase (Worthington Biochemical Corporation, Freehold, New Jersey, U.S.A.) in weak ammonia; both were filtered through Chamberland (Dalton P. 5) candles. The media were distributed in 6 in. \times $\frac{5}{8}$ in. tubes in 4 ml. quantities.

The organisms used were a strain of *Mycobacterium tuberculosis* H37Rv and a strain of B.C.G. received originally from Dr R. J. W. Rees (National Institute for Medical Research, Mill Hill, London N.W. 7). These cultures were maintained in Dubos medium or on Löwenstein-Jensen slopes (obtained from Southern Group Laboratory, Park Hospital, Hither Green, London, S.E. 13); in most experiments control cultures in Dubos medium were set up. Isoniazid-resistant variants of these two strains were obtained by subculture from isoniazid-containing Dubos cultures. Both these derived resistant strains were resistant, in Dubos medium, to about 10 μ g. isoniazid/ml., whereas the parent cultures were sensitive to about 0.05 μ g. isoniazid/ml. or less.

The inoculum was 0.02–0.04 ml. of culture/ml. medium. For strain H37Rv, 7–10-day cultures and, for B.C.G., 10–14-day cultures in Dubos & Davis (1946) medium were used. Cultures were incubated at 37° and growth was observed at intervals. No attempt was made to give more than a rough estimate of the amount of growth since the type of growth varied with different media; it was granular in the absence of Tween 80 but diffuse in its presence.

RESULTS

Effect of haemin on growth

Table 1 shows that in Fisher's medium the isoniazid-sensitive strain (S) of H37Rv grew well after 8 days of incubation, but that the isoniazid-resistant strain (R) grew only in the presence of haemin, down to a concentration of

c. 1 $\mu\text{g./ml.}$ The effect of haemin was essentially the same in Fisher's medium alone and with the addition of albumin (0.35 %) or Tween 80 (0.05 %) or both together. In some experiments the resistant strain did grow in the absence of haemin, but always more slowly and much less profusely than the sensitive strain. The action of albumin and of Tween 80 was variable. In some experiments albumin stimulated the growth of the R strain, whereas Tween 80 stimulated the S strain. The effect of haemin, however, was consistent, and this and many other experiments fully confirm Fisher's experimental facts.

Table 1. *Effect of haemin on growth of Mycobacterium tuberculosis H37 Rv, isoniazid-sensitive (S) and isoniazid-resistant (R) strains in Fisher's medium*

		Eight days of incubation								
		Added haemin ($\mu\text{g./ml.}$)								
		100	50	25	12.5	6.25	3.1	1.5	0.75	0
Medium	Isoniazid sensitivity	Relative growth								
Fisher	S	+	+	+	+	+	+	+	+	+
	R	+	+	+	+	+	+	+	0	0
Fisher + albumin	S	+	+	+	+	+	+	+	+	+
	R	+	+	+	+	+	+	+	+	0
Fisher + Tween 80	S	+	+	+	+	+	+	+	+	+
	R	+	+	+	+	+	+	0	0	0
Fisher + albumin + Tween 80	S	+	+	+	+	+	+	+	+	+
	R	+	+	+	+	+	+	0	0	0

+ = good growth; 0 = no growth.

Table 2. *Effect of haemin on growth of Mycobacterium tuberculosis strain B.C.G. in Fisher's medium; comparison of isoniazid-sensitive (S) and isoniazid-resistant (R) strains*

		Eight days of incubation.								
		Added haemin ($\mu\text{g./ml.}$)								
		100	50	25	12.5	6.25	3.1	1.5	0.75	0
Medium	Isoniazid sensitivity	Relative growth								
Fisher	S	0	0	0	0	0	0	0	0	0
	R	0	0	0	0	0	0	0	0	0
Fisher + albumin	S	+	+	+	+	+	+	+	+	+
	R	tr.	\pm	\pm	\pm	\pm	\pm	tr.	0	0
Fisher + Tween 80	S	0	0	0	0	0	0	0	0	0
	R	0	0	0	0	0	0	0	0	0
Fisher + albumin + Tween 80	S	++	++	++	++	++	+	+	+	+
	R	\pm	+	+	+	+	\pm	tr.	tr.	0

+, ++ = good growth; \pm = moderate growth; tr. = trace growth; 0 = no growth.

Table 2 shows that neither the S nor the R strains of B.C.G. grew in Fisher's medium unless albumin was added. The S strain grew well in its

presence and profusely in the presence of albumin + Tween 80. The R strain in addition to albumin required haemin (1.5–3 $\mu\text{g./ml.}$).

The effect of varying the concentration of Tween 80 on the growth of H37 Rv, R strain, was next investigated. Low concentrations of Tween 80 produced erratic results, but as the concentration of Tween 80 increased, a higher concentration of haemin was needed for growth of the R strain to occur. The results of one experiment are shown in Table 3.

Table 3. *Effect of Tween 80 and haemin on growth of isoniazid-resistant (R) Mycobacterium tuberculosis strain H37 Rv*

Tween 80 (%)	Added haemin ($\mu\text{g./ml.}$)			
	50	10	1	0
0.0005	+	+	+	0
0.005	+	+	0	0
0.05	+	+	0	0
0.5	+	+	0	0
5.0	\pm	0	0	0

+ = good growth; \pm = moderate growth; 0 = no growth.

Table 4. *Effect of haemin and Tween 80 on isoniazid-sensitivity of Mycobacterium tuberculosis strain H37 Rv*

		Sensitive (S) strain						Resistant (R) strain					
		Concentration of isoniazid ($\mu\text{g./ml.}$)											
Tween 80 (%)	Conc. of haemin ($\mu\text{g./ml.}$)	100	10	1	0.1	0.01	0	100	10	1	0.1	0.01	0
A	None	0	0	0	0	0	+	0	0	0	0	0	0
		10	0	0	+	+	+	+	0	+	+	+	+
		50	0	+	+	+	+	+	0	+	+	+	+
B	0.0005	0	0	0	0	0	+	0	0	0	0	0	0
		10	0	0	+	+	+	+	0	+	+	+	+
		50	0	±	+	+	+	+	0	+	+	+	+
C	0.005	0	0	0	0	0	+	0	0	0	0	0	0
	0.05	10	0	0	+	+	+	+	0	+	+	+	+
	0.5	50	0	0	+	+	+	+	0	+	+	+	+
D	5.0	No growth of S or R strain in absence or presence of haemin											

Antagonism of isoniazid by haemin

Table 4A shows an example of the powerful antagonistic effect of haemin on isoniazid sensitivity described by Fisher (1954a). The S strain of H37 Rv in Fisher's medium was inhibited by 0.01 $\mu\text{g.}$ isoniazid/ml., but in the presence of 10 $\mu\text{g.}$ haemin/ml. it grew in 1 $\mu\text{g.}$ isoniazid/ml., and in the presence of 50 $\mu\text{g.}$ haemin/ml. it grew in 10 $\mu\text{g.}$ isoniazid/ml. In this it was indistinguishable from the R strain which grew in 10 $\mu\text{g.}$ isoniazid/ml. in the presence either of 10 or 50 $\mu\text{g.}$ haemin/ml., but failed to grow at all in the basal medium alone. These results again confirm Fisher's.

The effect of varying the concentration of Tween 80 on the isoniazid-antagonizing effect of haemin was also examined. Table 4 shows that the effectiveness of haemin in antagonizing the action of isoniazid on the S strain of H37Rv was decreased as the concentration of Tween 80 was increased. The R strain of H37Rv grew in 10 μ g. isoniazid/ml. so long as haemin was present, in all concentrations of Tween 80 and in its absence.

Table 5. *Effect of haemin on isoniazid sensitivity of Mycobacterium tuberculosis strain B.C.G. (S)*

Fifteen days of incubation; haemin concentration 100 μ g./ml.; albumin 0.35 %; Tween 80 0.05 %.

Medium	Concentration of isoniazid (μ g./ml.)							
	40	8	1.6	0.32	0.06	0.01	0.002	0
Fisher	0	0	0	0	0	0	\pm	\pm
Fisher + haemin	0	0	\pm	\pm	\pm	\pm	\pm	\pm
Fisher + haemin + albumin	0	+	+	+	+	+	+	+
Fisher + haemin + Tween 80	0	0	0	0	0	0	0	0
Fisher + haemin + albumin + Tween 80	0	++	++	++	++	++	++	++

+, ++ = good growth; \pm = moderate to poor growth; 0 = no growth.

Table 5 shows that the S strain of B.C.G. which was sensitive to 0.01 μ g. isoniazid/ml. in the absence of haemin, grew in 1.6 μ g. isoniazid/ml. in the presence of haemin (100 μ g./ml.), and in 8 μ g. isoniazid/ml. in the presence of haemin + albumin, and of haemin + Tween 80 + albumin. The combination of Tween 80 and haemin, in the absence of albumin, was inhibitory to the S strain which failed to grow even in the control medium without isoniazid.

Fisher (1954*a*), working with H37Rv, found that bovine serum or albumin almost completely nullified the haemin/isoniazid antagonism and suggested that only 'free' haemin could act as an isoniazid antagonist. Although this point has not yet been further investigated with H37Rv, the above-mentioned results show that with B.C.G. at any rate, albumin (0.35 %) added to the medium did not interfere with haemin/isoniazid antagonism. On the other hand, a crude preparation containing haemoglobin, consisting of lysed sheep red cells, showed only slight isoniazid antagonism but itself interfered with the isoniazid-antagonizing effect of haemin. This problem is being investigated further, both for its intrinsic interest and because it clearly has an important bearing on the interpretation of isoniazid sensitivity tests performed in media containing blood or lysed blood.

It is known in uninoculated media that isoniazid may be inactivated in the presence of haemin (Youmans, personal communication; Cohn, Oda, Kovitz & Middlebrook, 1954). When isoniazid sensitivity tests were performed on 7-day cultures of the S strain of B.C.G. which had grown in 10 μ g. isoniazid/ml. (in the presence of haemin), the organism showed normal sensitivity to less than 0.05 μ g. isoniazid/ml. Corresponding tests on the R strain showed it to be normally resistant to about 10 μ g. isoniazid/ml., again in agreement with

Fisher (1954*a*). When, however, sensitivity tests were performed on these same cultures after 3 weeks of incubation in the medium containing 10 μ g. isoniazid/ml. together with haemin, it was found that some selection of resistant organisms had occurred. This indirectly suggests that the concentration of isoniazid, even in the presence of haemin, was high enough at any rate to maintain the selection pressure necessary for the emergence of resistant organisms and gives support to the view that the isoniazid-antagonizing effect of haemin is not entirely due to chemical inactivation of isoniazid.

Influence of changes in nitrogen source

In all the experiments so far described, the medium used was made up according to Fisher's formula, and in this medium the growth-promoting and isoniazid-antagonizing effects of haemin described by Fisher were repeatedly confirmed. In the medium of Dubos & Davis (1946), however, differences in growth requirements between the isoniazid-sensitive and isoniazid-resistant strains of H37 Rv and of B.C.G. were not consistently observed. Table 6 shows one of several experiments in Dubos & Davis medium in which no difference between the two strains was detected. The composition of the two media is shown in Table 7; the main differences (apart from the concentration of the magnesium sulphate) are in the nitrogen and carbon sources. Some preliminary experiments have been performed with different nitrogen sources in Fisher's medium alone and with the addition of albumin, Tween 80 and haemin separately and together. The results may be briefly summarized.

Table 6. *Growth of Mycobacterium tuberculosis strain B.C.G. isoniazid sensitive (S) and resistant (R) strains in Dubos & Davis medium*

21 days of incubation; added haemin = 100 μ g./ml.

Medium	Strain S		Strain R	
	No haemin	With haemin	No haemin	With haemin
	Relative growth			
Dubos	0	0	0	0
Dubos + albumin	+	+	+	+
Dubos + Tween 80	+	0	+	0
Dubos + albumin + Tween 80	+++	+++	+++	+++

+, +++ = good growth; 0 = no growth.

In Fisher's medium with the addition of albumin and Tween 80, the S strain of B.C.G. grew well and profusely with all the nitrogen sources tested and gave the usual diffuse type of growth, both with and without haemin. The R strain also grew well in this medium with and without haemin, with ammonium phosphate, ammonium sulphate or glycine as nitrogen source, but with asparagine as nitrogen source this R strain grew only in the presence of haemin (Table 8). In Fisher's medium with albumin but without Tween 80 haemin had a similar effect though the growth of both strains was slower and, as usual

in the absence of Tween 80, granular. Fisher's medium with Tween 80 but without albumin gave erratic results in different experiments. In one experiment with casein hydrolysate as nitrogen source in Fisher's medium the R strain in the presence of albumin + Tween 80 grew well without haemin but required it when albumin was omitted. This experiment suggests that the difference in behaviour of the R strain as between Fisher's and Dubos & Davis medium may be partly due to the different nitrogen source. It is clear that the apparent haemin dependence of the R strain is itself affected by changes in the composition of the medium.

Table 7. *Composition of Fisher and Dubos & Davis (1946) media*

	Fisher	Dubos & Davis
	(g./l.)	
Potassium dihydrogen phosphate	1.00	1.00
Disodium hydrogen phosphate	6.30	6.25
Sodium citrate	1.50	1.50
Magnesium sulphate (MgSO ₄ .7H ₂ O)	0.20	0.60
Asparagine	1.00	0
Glycerol	20.00	0
	(ml./l.)	
Casein hydrolysate (acid extraction) 20 % solution	0	10.0
Tween 80 10 % solution	0	5.0
Albumin (9 % fraction V, Armour and Co.)	0	40.0
Final pH value	6.8	7.2

Table 8. *Effect of nitrogen source on haemin 'dependence'. Mycobacterium tuberculosis strain B.C.G., isoniazid-sensitive (S) and isoniazid-resistant (R) strains*

Eighteen days of incubation; haemin, when added, at 100 µg./ml.

		Nitrogen source (about 0.005 M)							
		Asparagine		Ammonium phosphate		Ammonium sulphate		Glycine	
		Haemin							
		Relative growth							
Medium	Sensitivity of strain	-	+	-	+	-	+	-	+
Fisher	S	0	0	0	0	0	0	0	0
	R	0	0	0	0	0	0	0	0
Fisher + albumin	S	++	++	++	++	++	+	+	+
	R	tr.	± ±	+	+	+	+	+	+
Fisher + Tween 80	S	0	0	0	0	0	0	0	0
	R	0	0	0	+	±	±	0	0
Fisher + albumin + Tween 80	S	+++	+++	+++	+++	+++	+++	+++	+++
	R	0	+++	+++	+++	+++	+++	+++	+++

+, ++, +++ = good growth; ± = moderate growth; tr. = trace growth; 0 = no growth.

The effect of catalase

Middlebrook, following up his observation that isoniazid-resistant tubercle bacilli are deficient in catalase (Middlebrook, 1954), found that the addition of catalase to deficient culture media permitted growth of these organisms (Middlebrook, personal communication). Table 9 shows a comparison of the effects of a filtered catalase solution and of haemin on the growth of S and R strains of H37Rv in Fisher's medium (with asparagine). In all the media tested the S strain grew well and was not stimulated either by haemin or catalase. The R strain grew only in the presence of haemin or catalase. Similar results were obtained with B.C.G. The concentrations required were for catalase *c.* 10^{-10} – 10^{-11} M, for haemin *c.* 10^{-5} – 10^{-6} M. The efficiency of catalase in promoting the growth of the R strain was therefore *c.* 100,000 times that of haemin. This strongly suggested that the role of haemin in promoting the growth of isoniazid-resistant tubercle bacilli in Fisher's medium was, in fact, one of destroying peroxide. Further support for this view was given by an experiment which showed that whereas the growth-promoting effect of haemin was not decreased by boiling for 3 min., the growth-promoting effect of catalase was decreased to about the degree which would be expected from its haemin content.

Table 9. *Effect of haemin and catalase on growth of Mycobacterium tuberculosis strains H37Rv isoniazid-sensitive and isoniazid-insensitive (S and R) in Fisher's medium*

		Ten days of incubation.											
		Catalase concentration							Haemin concentration				No addi- tion
		4 M ×							1.6 M ×				
		10 ⁻⁸	10 ⁻⁹	10 ⁻¹⁰	10 ⁻¹¹	10 ⁻¹²	10 ⁻¹³	10 ⁻¹⁴	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	
Medium	Sensitivity of strain	Relative growth											
Fisher	S	+	+	+	+	+	+	+	+	+	+	+	+
	R	+	+	+	0	0	0	0	+	+	0	0	0
Fisher + albumin	S	+	+	+	+	+	+	+	+	+	+	+	+
	R	+	+	+	+	0	0	0	+	+	+	+	0
Fisher + Tween 80	S	++	++	++	++	++	++	++	++	++	++	++	++
	R	±	+	+	0	0	0	0	+	+	0	0	0
Fisher + albumin + Tween 80	S	++	++	++	++	++	++	++	++	++	++	++	++
	R	+	+	+	±	0	0	0	+	+	±	0	0

+, ++ = good growth; ± = medium growth; 0 = no growth.

DISCUSSION

Any attempt to explain the action of isoniazid on mycobacteria must account for its specificity in at least three senses: its specificity for mycobacteria as compared with other micro-organisms, the specific differences between sensitive and resistant organisms, and specific differences between isoniazid and other drugs. Most workers have followed two lines of inquiry: the search for

a specific metabolite capable of antagonizing isoniazid and the search for specific differences in metabolism or growth requirement between sensitive and resistant organisms. Many examples of antagonism to isoniazid have been described, but Fisher's work with haemin was the first that seemed to offer a real clue to the problems of specificity. This clue was strengthened both by Middlebrook's demonstration that isoniazid resistance is associated with a specific deficiency in catalase in the resistant organisms and by the discovery that haemin 'dependence' is itself dependent upon the exact composition of the growth medium.

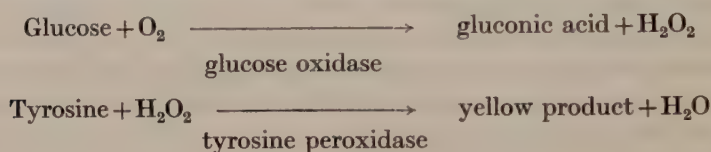
Middlebrook's work on catalase at once suggested that in Fisher's medium haemin was acting in a 'catalatic' role, destroying H_2O_2 . It is well known that 'catalase' activity of increasing efficiency is shown by a number of iron-containing substances from iron itself, through iron salts and haemin, to peroxidases and catalase. Many of the facts at present known about isoniazid-resistant organisms become intelligible if we adopt the following simple theory—that *Mycobacterium tuberculosis* is sensitive to low concentrations of H_2O_2 (whether present as the result of chemical changes in the medium or produced by the organisms themselves), that growth will continue only when H_2O_2 does not accumulate, that isoniazid-resistant organisms, being deficient in catalase, cannot remove H_2O_2 , that isoniazid blocks the removal of H_2O_2 by sensitive organisms and that the addition of any substance with a catalase-like action would not only enable isoniazid-resistant organisms to grow but would antagonize the action of isoniazid on sensitive organisms. This reasoning, besides accounting for Fisher's and Middlebrook's experimental results, could also explain the observations of Barry, Conalty & Gaffney (1953) that isoniazid-resistant tubercle bacilli could grow in a chemically-defined medium provided the medium had been previously treated with charcoal, and some observations in this laboratory on the effect of iron on the growth of isoniazid-resistant tubercle bacilli in Fisher's medium. It could even account for the confusing results obtained in media containing Tween 80, since detoxication of long chain fatty acids might well be due to removal of toxic peroxides by substances such as haemin with catalase or peroxidase activity. The importance of similar mechanisms has been shown in other groups of organisms by McLeod & Gordon (1925 *a, b*), by Herbert (1949) in *Pasteurella pestis*, by Jordan (1952 *a, b*) in *P. septica* and by Proom, Woiwod, Barnes & Orbell (1950) in *Shigella dysenteriae*.

Further evidence in support of this theory is given by some experiments in this laboratory on the effect of added H_2O_2 on tubercle bacilli. We have found that isoniazid-resistant organisms appear much more sensitive to H_2O_2 than isoniazid-sensitive organisms, as judged both by exposure to its action followed by subculture and by inhibition of growth in several different media containing falling concentrations of H_2O_2 . An account of these experiments is being prepared.

This theory involves the specific assumption that in isoniazid-sensitive organisms isoniazid affects an enzyme or group of enzymes which are lacking in the resistant organisms, an assumption for which there is much experimental

support with other drugs (Julius, 1952). The evidence so far suggests that isoniazid-resistant tubercle bacilli are vulnerable to peroxides partly at least because they lack the capacity to produce catalase or peroxidases. If we accept the corollary that it is precisely this defect which makes them resistant to isoniazid, and that isoniazid affects sensitive organisms by interfering with their capacity to destroy peroxides, then we might expect to find that isoniazid inhibits the action of catalase. We have found in this laboratory, however, by manometric methods that this is not so; the activity of catalase from various sources was unaffected by isoniazid. This suggests either that isoniazid inhibits the formation of catalase rather than affects its activity or that the effect of isoniazid is not so much on catalase itself as on other peroxidases, perhaps specific to mycobacteria.

W. E. Knox (1954) showed that in certain enzyme preparations coupled oxidation of glucose and tyrosine or tryptophan can occur, but only under highly specific conditions as shown diagrammatically:



Continued oxidation of glucose occurs if H_2O_2 , as it is formed, is destroyed by catalase; but oxidation of tyrosine in this system occurs only when a specific tyrosine peroxidase is present as well. Similar coupled oxidations with lactic oxidase and *p*-aminobenzoic acid have been described in mycobacteria by Edson & Cousins (1953). These enzymes may well be adaptive in nature. It is known, for example, that formation of catalase (Chantrenne & Courtois, 1954) and of cytochrome peroxidase (Chantrenne, 1954) is an adaptive change induced in the presence of oxygen. It is at least possible that in the coupled oxidations referred to above, oxygen induces the formation of an oxidase, which oxidizes the first substrate (e.g. glucose) with formation of H_2O_2 , and that the H_2O_2 , formed itself catalytically, induces the formation of a catalase or peroxidase which then destroys it, just as for example penicillin in very low concentrations induces the formation of penicillinase in *Bacillus cereus* (Pollock, 1950). In the presence of a second substrate and a specific peroxidase, a coupled oxidation occurs. In this way a series of enzymes would be successively formed, but only if H_2O_2 did not accumulate.

This leads back to the problem of specificity. Many other micro-organisms, whether or not dependent upon haemin, are not sensitive to isoniazid, so there must be some specific point in the peroxide-destroying sequence of enzymes which is attacked by isoniazid. The evidence given above suggests that perhaps the specific point may be the substrate asparagine, and that isoniazid-resistant organisms are unable to oxidize asparagine because they do not possess a specific asparagine peroxidase which is present in isoniazid-sensitive organisms. In these the formation of the enzyme is blocked because isoniazid competes with asparagine which is sterically and electronically similar. Some

such theory would at least explain why isoniazid-resistant strains appear to require haemin in the presence of asparagine and not of other nitrogen sources, by postulating that isoniazid is competing with a specific substrate, asparagine, rather than competing with an active centre on a group of enzymes such as catalase and peroxidases which are widely distributed in biological materials. Whether such a view is correct or not in detail, it seems clear that the action of isoniazid is closely connected with certain peroxide-destroying mechanisms and that isoniazid-resistant organisms are deficient in such mechanisms whose integrity is essential for survival and growth in certain types of media in which asparagine is the nitrogen source. Oxidation of unsaturated fatty acids such as linoleic, linolenic and arachidonic acids by lipoxidase is known to be associated with the production of peroxides (Holman & Bergström, 1951), and the action of various drugs on mycobacteria is known to be greatly influenced by the presence of Tween 80 and other substances which contain unsaturated fatty acids. It is even possible that differences in the peroxidase or catalase activity of isoniazid sensitive and resistant tubercle bacilli may provide a clue to the confusing dual action of this class of compound on mycobacteria—since substances which are oxidized with production of peroxides might stimulate growth when H_2O_2 was removed but might be lethal if it accumulated.

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Chemically Induced Aberrations of Mitosis in Bacteria

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SUMMARY: *Bacillus megaterium* was studied cytologically during exposure to a number of toxic agents, including known mitotic inhibitors. Many antibiotics and sodium *p*-aminosalicylate at inhibitory concentrations induced an increase in the size and optical density of the stained nuclei, and a preponderance of configurations resembling metaphase and anaphase stages. Continued chromosome reduplication results in the establishment of a transient polyploidy. Isoniazid and benzimidazole bring about a decrease in the amount of stainable material in the nucleus. Penicillin and bacitracin produce no obvious changes in the nuclear pattern in this organism. The significance of the aberrations observed is discussed. Further support for the similarity of bacterial nuclei to the nuclei of higher organisms is provided.

In most of its fundamental aspects the bacterial cell seems to be very similar to the cells of higher plants and animals. Despite the establishment of similarities in biochemistry, physiology, and the mechanisms which control heredity in bacteria and larger cells, the cytology of the bacterial cell remains partially controversial. The existence of nuclei in the bacterial cell was proven only during the last two decades, but the nuclear structure and divisional mechanism are still subjects of discussion. Technical and interpretative difficulties are in large part responsible for this situation. The chromosomal components of even the larger bacteria have dimensions which lie at the limit of resolution of the light microscope. In consequence, these structures can be seen with only limited clarity as discrete entities even when their orientation is optimal. Technical problems, such as the presence of masking ribonucleic acid (RNA) in the cytoplasm and the susceptibility of the cell structure to distortion by fixatives, drying, and room temperature dehydration, have been the source of much confusion.

Reliance on analogy with higher forms has been used as a basis of criticism by Lamanna & Mallette (1953) in their consideration of the exposition of mitotic processes in bacteria by DeLamater (1953). Obviously our detailed knowledge of the mitotic cycle could not have been formulated through investigations on the bacterial cell, because of its small size. However, a belief that filamentous or rod-like structures in the bacterial nucleus are similar in nature to the more easily observed chromosomes of higher organisms would be strengthened if the alleged analogy of Lamanna & Mallette holds true under

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a variety of different experimental conditions. An answer to the question, 'Do these similar structures in the bacterial nucleus behave similarly?' may thus be arrived at.

The study of mitosis in bacteria under the action of so-called mitotic poisons and other inhibitors represents an alternative set of conditions, and might supply additional evidence for the reality of mitotic configurations in the bacterial nucleus. Levan (1953) wrote: 'If similar deviations were induced in mitosis of yeast as were known to occur in higher plants after such (c-mitotic) treatment, it would be an indication that the Feulgen-positive bodies observed in yeast were really chromosomes undergoing mitosis.' (Levan (1954) suggested the use of the term c-mitotic 'in a broad sense for all morphologically discernible disturbance of the spindle function irrespective of any notions as to the mode of action underlying the effects...'.) Similar reasoning may be used for bacteria. In addition to this theoretical implication, it will be shown here that the action of certain toxic substances provides the following technical advantages: (1) the percentage of cells with mitotic figures increases from a small fraction to almost 100 %, thus eliminating the necessity of selecting single cells for the demonstration of metaphase spindles in bacteria; (2) the optical density of stained nuclear material is increased. The present study deals with the general cytological effect of several antibiotics, sodium *p*-aminosalicylate, isoniazid and the purine analogue benzimidazole.

METHODS

Organisms. *Bacillus megaterium*, the strain employed by DeLamater & Hunter (1951), was used as the test organism. Mutants resistant to high concentrations of the toxic agents (Szybalski, 1954) were exposed to the given agents, together with wild type.

Media. Suspensions of up to 6×10^8 cells/ml. were prepared by growing the organisms in double strength nutrient broth (Difco), 16 g. desiccated medium/l., for 6 hr. at 34° in small bottles with agitation. The agar medium had the following composition: nutrient broth desiccated (Difco), 8 g.; yeast extract (Difco), 1 g.; NaCl, 5 g.; NZ amine A (a peptone supplied by 'Sheffield Farms', Sheffield Chemical Co., Norwich, New York, division of National Dairy Products Inc.), 5 g.; agar 20 g.; demineralized water, 1 l.

Methods of application of toxic substances. Appropriate volumes of solution were added to 10 ml. portions of melted agar medium in $\frac{1}{2}$ oz. screw-cap bottles and mixed. Plates were prepared according to the gradient plate method of Szybalski (1952). This method was used because it permitted the study of the action of the toxic agent over a wide range of concentrations. The agent was incorporated only into the upper wedge of agar, providing approximately a tenfold range in concentration along the gradient. The concentration of the toxic agent was adjusted to demonstrate both growth and inhibition on the same plate. The figures indicating concentration ranges in the following list have the following significance: 1/0, indicates that 1 μ g./ml. of toxic substance was added to the upper wedge of agar, and none (0 μ g./ml.) to the lower wedge.

The concentration on such a plate varies from about 1 to 0.1 $\mu\text{g./ml.}$ along the axis of the gradient. The following concentration ranges ($\mu\text{g./ml.}$) were employed: oxytetracycline hydrochloride (tetracycline), 2/0 and 1/0; chlor-tetracycline hydrochloride (aureomycin), 0.5/0; streptomycin sulphate, 3/0; sodium *p*-aminosalicylate, 2000/0; carbomycin, 4/0; erythromycin, 0.5/0; neomycin sulphate, 0.1/0; viomycin sulphate, 40/0; chloramphenicol, 5/0; potassium penicillin, 1000/0; bacitracin, 0.2/0; isoniazid, 2000/0; benzimidazole, 2000/0.

After pre-drying of the surface, the suspension of organisms was streaked over the agar with a pipette in about five streaks parallel to the axis of the gradient. The plates were then incubated and the organisms examined cytologically after various periods of exposure. Any resistant colonies were cut out from the agar before making smears. Experiments with different concentrations of agents, time of action and recovery were all done on a single plate, thus eliminating a major source of variation. All strains were examined under comparable conditions in the absence of any toxic agent.

Termination of exposure. Recovery of the organisms from the inhibitory action of certain toxic agents was permitted by either of the two following procedures: (1) awaiting spontaneous decay of certain antibiotics; (2) superimposing on plain agar the strip of agar bearing the streak on its upper surface, thus permitting the toxic agent to diffuse downward and decrease the concentration at the level of the bacterial cells.

Cytological procedure. A strip of agar bearing a streak of organisms and extending along the entire gradient was cut out. This was divided into four portions of equal length, corresponding to increasing concentration of toxic agent. Following fixation of the cells in the vapour of OsO_4 (1% solution) for 3 min., smears were made on cover-slips marked so as to make possible identification of the range and direction of increase of the drug concentration. The cells were then hydrolysed in *N*-HCl, stained in thionin- SO_2 , and dehydrated in the cold according to the method of DeLamater (1951).

Photomicrography. Observations were made with a Bausch & Lomb research microscope equipped with an achromatic condenser of 1.4 N.A., a $\times 90$ apochromatic oil-immersion objective of 1.4 N.A., and $\times 12.5$ compensating oculars. Photographs were taken with a Bausch & Lomb L camera on Kodak Panatomic-X film, using a camera length of 20 in. A Bausch & Lomb interference filter of wave-length 570 $\text{m}\mu$. provided maximum contrast. The final magnification of the illustrations is $\times 4500$.

RESULTS

The normal course of nuclear division in *Bacillus megaterium* was described by DeLamater & Mudd (1951) and interpreted as mitosis. Within each rod the nuclear divisions are more or less synchronous, but nuclei in interphase and in all stages of mitosis are found in every microscopic field. Metaphase and anaphase seem to be of shortest duration, as judged by the relative numbers of each stage and by comparison with several higher organisms in which the

length of the individual stages of the mitotic cycle is more easily defined. Exposure to the toxic agents produced one of the following gross effects on the optical density of stained nuclear material: (1) an increase in density (the majority of toxic agents tested); (2) no major change (bacitracin, penicillin); (3) a decrease in density (isoniazid, benzimidazole). Only in cases (1) and (2) was it practical and possible to detect and study induced aberrations in the nuclear divisional mechanism.

All the toxic agents comprising the following diverse group of drugs produced certain common effects: chlortetracycline, oxytetracycline, streptomycin, sodium *p*-aminosalicylate, neomycin, carbomycin, erythromycin, viomycin, chloramphenicol. The first effect was an increase in the optical density of stained nuclear material obtained in the vicinity of the inhibitory concentrations (see Szybalski, 1954) and at higher concentrations. Accompanying this change were aberrations in the nuclear divisional mechanism, manifested as an increased incidence of figures which were interpreted as metaphase and anaphase, and as possibly indicating an arrest at this stage of mitosis. When *Bacillus megaterium* was treated for 2 hr. at inhibitory concentrations or higher of oxytetracycline, chlortetracycline, sodium *p*-aminosalicylate, neomycin, carbomycin, erythromycin, or chloramphenicol, the percentage of nuclei in metaphase or anaphase approached 100 % (Pl. 1, fig. 1; Pl. 2, fig. 4; Pl. 3, figs. 6, 9; Pl. 4, figs. 10, 11). It was hardly possible to document this effect adequately by photographs because of limitations imposed by a short depth of the field. Below the inhibitory concentration the percentage of affected nuclei decreased very rapidly to zero, so that the thresholds of growth inhibition and the cytological effect were almost identical. Viomycin and streptomycin did not show similar effectiveness until after about 4 hr. (Pl. 2, fig. 5; Pl. 4, fig. 12). In the inhibited cells the bodies interpreted as centrioles were of increased size and optical density, and often gave evidence of reduplication (Pl. 4, figs. 10, 12).

With continued exposure the nuclei increased in size and density by elongation in the axis of the bacillus, becoming sausage-shaped (Pl. 1, fig. 2; Pl. 3, fig. 7). Within such condensed masses individual structures resembling chromosomes could not usually be defined. In occasional nuclei it was possible to count more than the haploid number of such chromosomes (Pl. 2, fig. 4; Pl. 3, fig. 7). These observations suggested that deoxyribonucleic acid (DNA) synthesis and chromosome reduplication were continuing in the absence of nuclear division. The term polyploidy is used here and in two previous papers (DeLamater, 1953; DeLamater, Hunter, Szybalski, Minsavage & Bryson, 1953) to describe this condition. However, because a resting polyploid nucleus is not reconstituted and perpetuated, as will be shown presently, the more explicit designation 'transient polyploidy' is fitting. It serves to distinguish the condition from the permanent polyploidy induced in higher forms by c-mitotic substances, e.g. colchicine.

Mutants of *Bacillus megaterium* resistant to higher concentrations of the toxic agents showed none of the described effects in the same range of concentration of the given agent.

In addition to differences in rapidity of action, other characteristics made it possible to distinguish certain of the toxic agents in this group by their cytological effects. Sodium *p*-aminosalicylate apparently effected an inhibition of centriolar migration, as evidenced by the unipolar nature of the spindle in many of the arrested nuclei (Pl. 4, fig. 11). This retardation of migration of the centrioles was also produced, but to a lesser degree, by other members of this group of agents. An interesting feature of cells arrested by carbomycin was the striking increase in dye affinity of the septum in bicellular rods (Pl. 3, fig. 6).

Recovery of *Bacillus megaterium* from the inhibitory effects was followed only after chlortetracycline and oxytetracycline treatment. While the toxic agents were being removed as described in Methods, the arrested cells were examined cytologically. A high percentage of the cells recovered. The sausage-shaped nuclear mass took on increasing length throughout an elongating cell, and at the same time lobulation, arising from constrictions in the nuclear membrane, became progressively apparent (Pl. 2, fig. 3). Within the individual lobules, of which there were as many as eight per nucleus, three contracted chromosomes, constituting the haploid complement, could be observed. The haploid nuclei, once separated, proceeded to completion of the mitotic process. With the re-institution of cell division, the picture became indistinguishable from the normal.

Isoniazid and benzimidazole. With respect to the optical density of stained nuclear material, these two toxic agents produced an effect opposite to that of the first group of inhibitors. There was a decrease in dye affinity of the chromosomes (Pl. 4, figs. 13, 14) to the extent that definition of detail was lost, and no aberrations in their structure or mechanism of separation could be detected. The cytological effect became apparent below the inhibitory concentrations, and increased in severity with increasing concentration of agent. In a proportion of cells isoniazid induced a striking increase in dye affinity of the cell membrane.

Penicillin and bacitracin. No obvious effects on the nuclear density or structure were noted during exposure of *Bacillus megaterium* to these compounds at any concentration on the gradients. Failure of penicillin to produce aberrations might have been due to the high penicillinase production of this organism.

DISCUSSION

The action of certain antibiotics on plant and animal cells has been studied by others and found to interfere with mitosis. Wilson (1950) and Wilson & Bowen (1951) noted characteristic mitotic upset in root tips of *Allium cepa* treated with streptomycin, neomycin, chlortetracycline, oxytetracycline, or chloromycetin; penicillin did not have any significant effect. One of the most striking deviations from the normal was 'failure of the metaphase-anaphase sequence'. Division figures, especially at prometaphase and metaphase, showed clumping and stickiness of the chromosomes, which were shorter than normal. Apparent delay in dissolution of the nuclear membrane was also noted. Recovery of *A. cepa* root tips was possible only when the toxic agent was chloramphenicol,

and no evidence of polyploidy was found. However, Wilson & Bowen stated that polyploidy is a theoretical possibility as a consequence of this c-mitotic arrest of normal mitosis.

Keilová (1948) and Keilová-Rodova (1950) studied the influence of streptomycin and chlortetracycline on the nuclei of cells in tissue culture, and observed a slight increase in the duration of metaphase and increased pyknosis and rhexis of chromosomes at metaphase. Woolley (1944) showed growth inhibition in yeasts and bacteria by benzimidazole. In chick tissue cultures exposed to this compound, Hughes (1952) reported an inhibitory effect on mitotic activity. Duncan & Woods (1953) treated onion root tips with benzimidazole and concluded that this substance inhibited both initiation of mitosis and DNA synthesis.

Studies on the effect of antibiotics on bacterial cytology have dealt mainly with penicillin, which has been shown to induce large body formation (see Dienes & Weinberger, 1951; or Tulasne, 1951, for a review of the literature). Where other antibiotics were also studied, they were usually reported to produce a very different effect from that of penicillin. The cytological effects of various antibiotics have been investigated by numerous workers (Tulasne, Vendrely & Minck, 1948; Werner & Kellenberger, 1948; Scanga, 1948*a, b*; Levaditi & Henry, 1948; Welsch, Nihoul & Demellenne-Jaminon, 1948; Eisenstark, Ward & Kyle, 1950; Preuner, von Prittwitz & Gaffron, 1951; Bringmann, 1952; Steinberg, 1952; Pulvertaft, 1952, 1953; DePoux, 1953; Kellenberger, 1953; Bergersen, 1953; Brieger, Cosslett & Glauert, 1953). Where nuclear phenomena were investigated, increase in size and density of the nuclei in the presence of chlortetracycline, oxytetracycline, or chloramphenicol was often reported (Steinberg, 1952; Kellenberger, 1953). Preuner, von Prittwitz and Gaffron concluded that nuclear division, but not cell division, was blocked by streptomycin. Bringmann noted that in the presence of this antibiotic *Escherichia coli* produced large amounts of nucleic acid on a medium on which nucleic acid production was usually minimal.

In the present study the toxic agents have been classified on the basis of their over-all effect on the optical density of stained nuclear material of *Bacillus megaterium*. Most of them produced a pronounced increase of optical density, while isoniazid and benzimidazole had the opposite effect. A possible parallel exists between this inverse action and the partial antagonism of streptomycin by isoniazid (Szybalski & Bryson, 1953) and by benzimidazole (Szybalski, unpublished).

Associated with the increase in stainable nuclear material is an inhibition of nuclear division, manifested as a rise in the relative number of stages which resemble anaphase and telophase mitotic configurations. So effective is this inhibition that the percentage of nuclei apparently in metaphase and anaphase approaches 100 %. The basis of the mitotic arrest may lie in malfunction of the spindle apparatus. Toxic agents which produce this effect, therefore, could be classified with the group of mitotic inhibitors which interferes with both anaphase movement and chromosome reconstruction in higher forms but, unlike colchicine, does not prevent the formation of the spindle.

It follows from the increase in nuclear size and optical density, and from counts of the putative chromosomes, that DNA synthesis and chromosome reduplication may proceed in *Bacillus megaterium* in the presence of toxic agents comprising one group of agents, even though mitosis be arrested. The resulting polyploid state persists only as long as the inhibitor is present, and is, therefore, the property of a nucleus in a stage of inhibited division. This is termed 'transient polyploidy' to distinguish it from the permanent polyploidy induced in higher forms by colchicine and certain other c-mitotic substances. Following early removal of the tetracyclines a majority of cells recovered from the bacteriostatic effect of these drugs. Restoration of the haploid state takes place directly by a process of reduction, microscopically observable as occurring by multiple constriction or sacculation of the nuclear membrane, which appears to persist throughout the mitotic cycle.

It is presumably no mere coincidence that agents known to inhibit mitosis in higher organisms may arrest nuclear division in almost 100 % of treated bacteria. The effect of benzimidazole indicates a further analogy between the nuclei of bacteria and those of higher forms, now evident under both normal and abnormal environmental conditions. The behaviour of the bacterial nucleus in the presence of known mitotic inhibitors thus provides supporting evidence for the existence of chromosomes in bacteria, and their distribution by a mitotic mechanism. For economy of both hypothesis and terminology, the concept of bacterial mitosis is again advanced as consistent with an increasing number of observations. Complete verification or refutation of this view will require the accumulation of extensive additional information, and may require further advances in technique and instrumentation. These analogies, both under normal conditions and in the presence of a number of inhibitors, together with the fact that the cytological effects of the inhibitors were demonstrated in nearly 100 % of the bacterial nuclei, seems to indicate that similarities in the nuclear division of bacteria and higher forms are more than coincidental.

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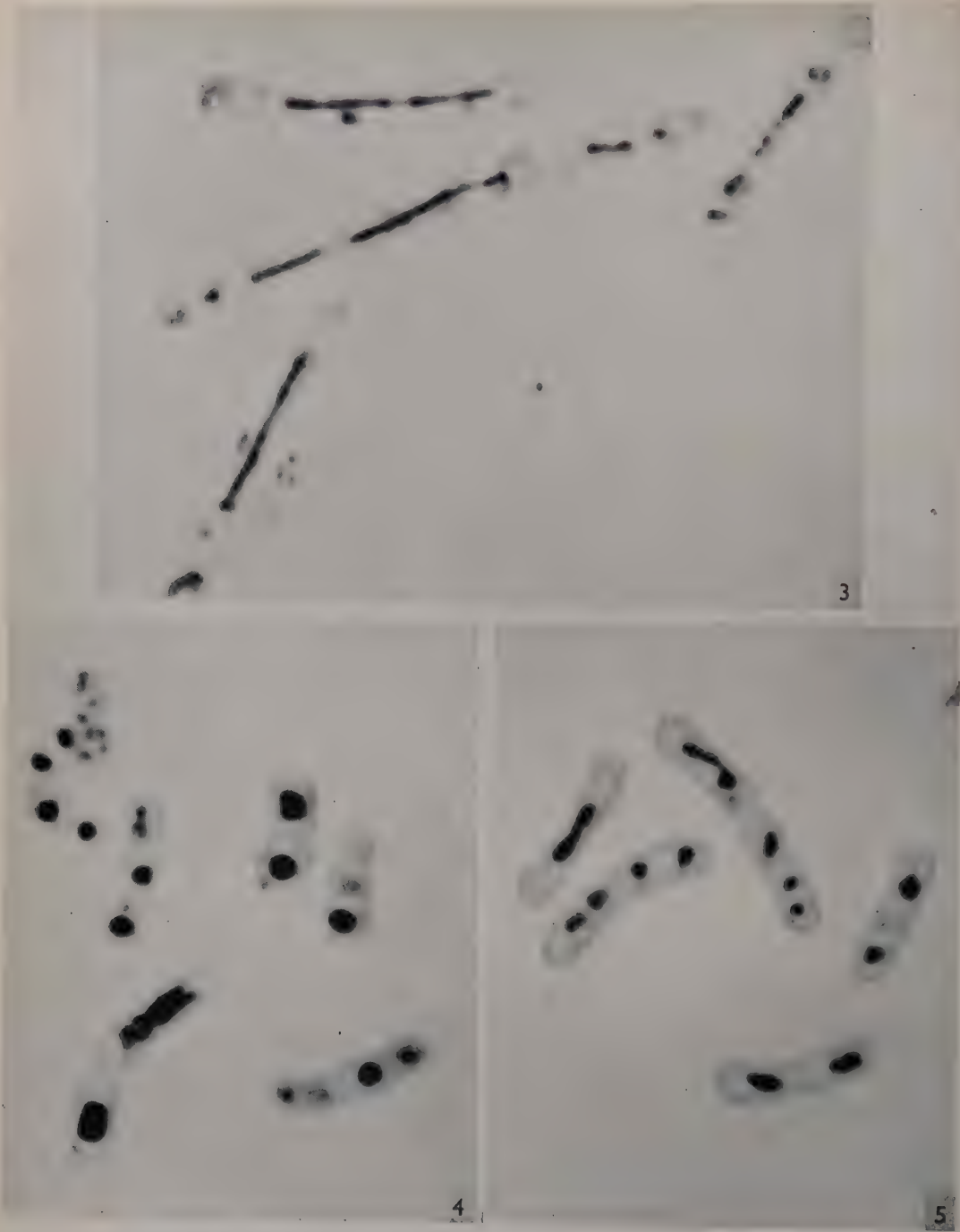
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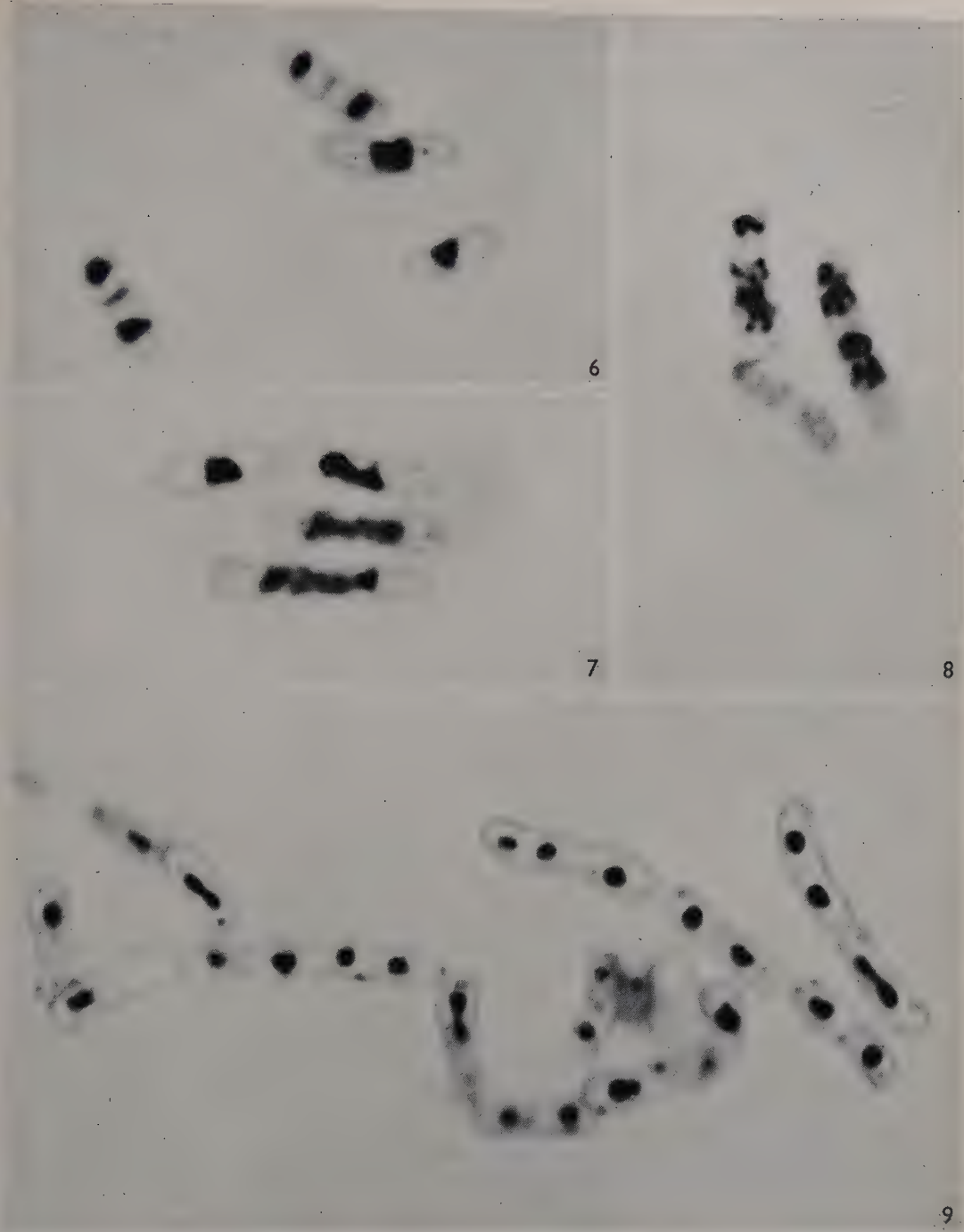


E. D. DeLAMATER *ET AL.*—CHEMICALLY INDUCED MITOTIC ABERRATIONS. PLATE 1

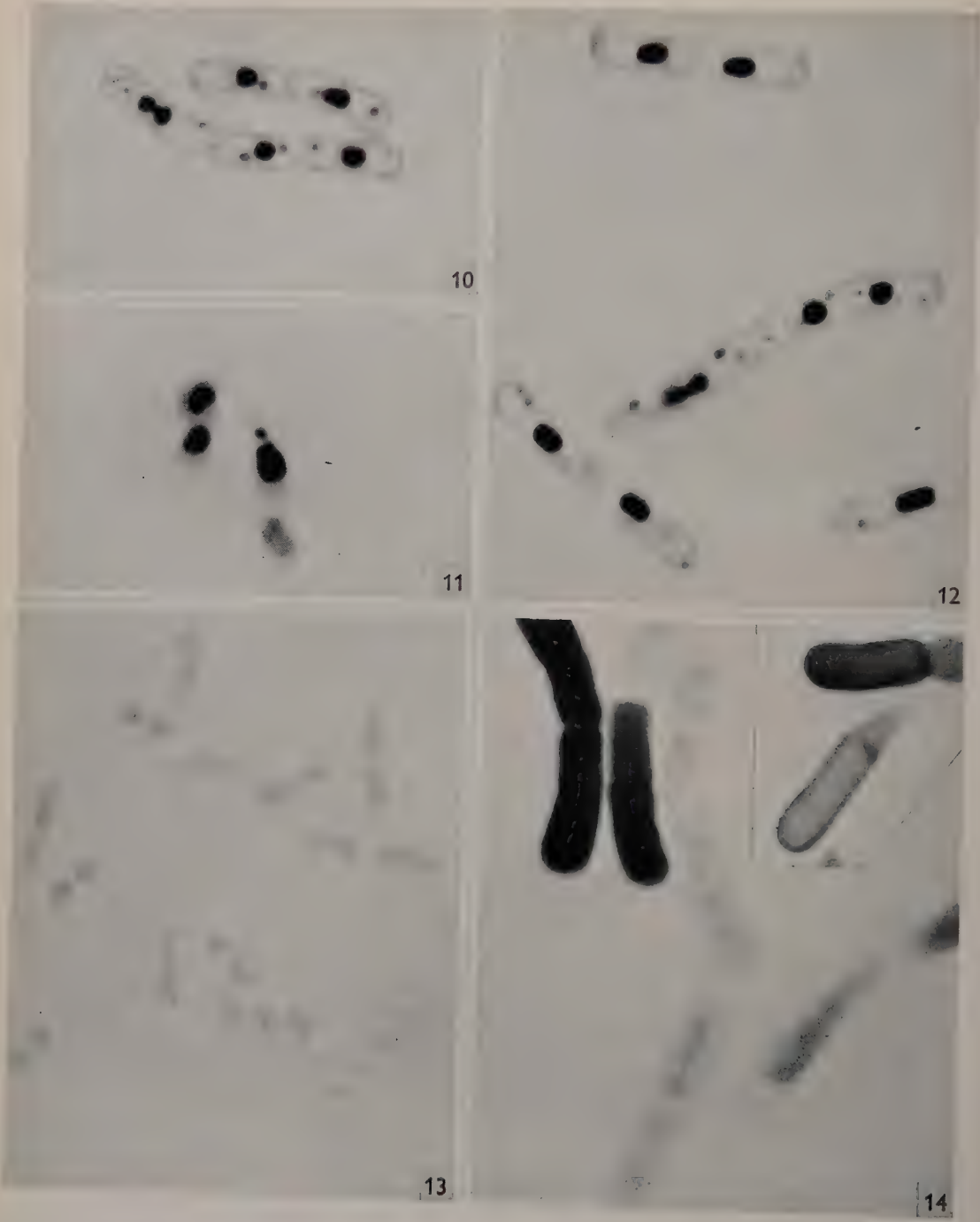
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E. D. DELAMATER *ET AL.*—CHEMICALLY INDUCED MITOTIC ABERRATIONS. PLATE 2



E. D. DeLAMATER *ET AL.*—CHEMICALLY INDUCED MITOTIC ABERRATIONS. PLATE 3



E. D. DeLAMATER *ET AL.*—CHEMICALLY INDUCED MITOTIC ABERRATIONS. PLATE 4

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EXPLANATION OF PLATES

PLATE 1. *Bacillus megaterium*, exposed to various toxic agents, stained by DeLamater's method. Magnification, $\times 4500$.

Fig. 1. 2 hr. exposure to inhibitory concentration of oxytetracycline. Increased optical density of stained nuclei in metaphase.

Fig. 2. 5 hr. exposure to inhibitory concentration of oxytetracycline. Elongation of nuclear mass.

PLATE 2. *Bacillus megaterium*, exposed to various toxic agents, stained by DeLamater's method. Magnification, $\times 4500$.

Fig. 3. 18 hr. exposure to oxytetracycline. Stages in recovery of cells due to spontaneous decay of antibiotic.

Fig. 4. 2 hr. exposure to inhibitory concentration of chlortetracycline. Cell in lower left shows stage in elongation of dense nuclear mass, in which more than three chromosomes are visible.

Fig. 5. 4 hr. exposure to inhibitory concentration of streptomycin.

PLATE 3. *Bacillus megaterium*, exposed to various toxic agents, stained by DeLamater's method. Magnification, $\times 4500$.

Fig. 6. 2 hr. exposure to inhibitory concentration of carbomycin. Heavily stained septa.

Fig. 7. 5 hr. exposure to inhibitory concentration of carbomycin. Elongation of arrested nuclei reveals multiplicity of chromosomal components.

Fig. 8. 4 hr. exposure to inhibitory concentration of erythromycin. Arrested nuclei show re-elongation of chromosomes.

Fig. 9. 2 hr. exposure to inhibitory concentration of neomycin.

PLATE 4. *Bacillus megaterium*, exposed to various toxic agents, stained by DeLamater's method. Magnification, $\times 4500$.

Fig. 10. 2 hr. exposure to inhibitory concentration of chloramphenicol.

Fig. 11. 2 hr. exposure to inhibitory concentration of sodium *p*-aminosalicylate. Unipolar spindle.

Fig. 12. 4 hr. exposure to inhibitory concentration of viomycin.

Fig. 13. 2 hr. exposure to inhibitory concentration of benzimidazole. Reduced dye affinity of nuclei.

Fig. 14. 2 hr. exposure to inhibitory concentration of isoniazid. Reduced dye affinity of nuclei. Increased dye affinity of membrane of several cells.

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Further Biological Properties of Trichothecin, an Antifungal Substance from *Trichothecium roseum* Link, and its Derivatives

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SUMMARY: Production of trichothecin in submerged aerated cultures of a strain of *Trichothecium roseum* is described. The antibiotic is active *in vitro* against certain fungi pathogenic in man, including the causative organisms of some generalized infections and several of the common skin pathogens. In general, the sensitive pathogens were inhibited at trichothecin concentrations of 16 p.p.m. or less and the antifungal activity was not influenced by the presence of blood serum (5%) in the medium. Trichothecin is toxic to laboratory animals; single doses at the rate of 250 mg./kg. intravenously in mice and subcutaneously in rats resulted in death. The antibiotic also produced a reaction when brought into contact with the skin.

The antifungal activity of a series of esters related to trichothecin was of the same order as that of the parent substance. With the exception of esters in which the ethylenic double bond of trichothecolone had been saturated, none of the known derivatives or fission products exhibits more than a very slight activity. The presence of a large excess of β -indolylacetic acid did not affect the antifungal activity of trichothecin. This suggests that the effect of the latter in inhibiting plant growth regulating activity of β -indolylacetic acid and related substances is not due to molecular combination of the two substances.

The presence of an antifungal compound in culture filtrates of *Trichothecium roseum* was reported by Brian & Hemming (1947), and the substance, trichothecin, responsible for the antifungal activity was isolated and some of its chemical and biological properties described by Freeman & Morrison (1949*a, b*). In addition to its antifungal action, trichothecin was reported by Hessayon (1951) to give well-marked stimulation of mycelial growth, at exceedingly small concentrations. Other metabolic products of the mould have been described by Robertson, Smithies & Tittensor (1949) and by Freeman, Morrison & Michael (1949). The activity of trichothecin as an inhibitor of plant virus infectivity and isolation from culture filtrates of *T. roseum* of a polysaccharide which behaved as an inhibitor, was described by Bawden & Freeman (1952). Freeman & Gill (1950) showed that trichothecin is the *isocrotonyl* ester ($C_{19}H_{24}O_5$) of the ketonic alcohol trichothecolone ($C_{15}H_{20}O_4$). Darpoux & Faivre-Amiot (1952) reported the presence in culture filtrates of *T. roseum* of a gummy substance (irritant substance) which gives rise to burns when placed on the skin. The presence of this irritant substance has been confirmed by the present author (unpublished observations), but it has not yet been obtained in a crystalline state. Yasue (1948, 1949) found that

a culture filtrate of *Cephalothecium roseum* (*T. roseum*) inhibited growth of *Staphylococcus aureus*. The active substance was not characterized, but Freeman, Morrison & Michael (1949) showed that rosein II had weak antibacterial activity.

The present paper is concerned with further biological properties of trichothecin including inhibition of growth of certain fungi pathogenic to man and toxicity in animals. The antifungal activity of various esters related to trichothecin and of some derivatives is reported.

EXPERIMENTAL

Preparation of trichothecin

Trichothecin was prepared from *Trichothecium roseum* strain no. F 292 in our laboratory collection (originally isolated at the London School of Hygiene and Tropical Medicine from tomato by Mrs Marcus; LSHTM catalogue no. 95). This strain gave maximum trichothecin yields of about 200 mg./l. in the culture filtrate after 21 days of incubation. The method of isolation closely followed that described by Freeman & Morrison (1949*a*); the most important modifications were: (a) addition of zinc sulphate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g./l.) to the culture medium which increased the yield of antibiotic and promoted improved sporulation of the fungus; (b) use of carbon tetrachloride, instead of chloroform, as a more specific solvent for extraction of antifungal substance from the culture filtrate. The crude residue from the carbon tetrachloride extract contained about 50% trichothecin; approximately half of this was recovered as the pure crystalline substance by the chromatographic method previously described.

Fermentations carried out with the above strain in submerged culture showed that trichothecin yields of 200 mg./l. were reached after 90 hr. incubation at 25° in a medium based on that of Freeman & Morrison (1949*a*) in which the initial pH value was adjusted to 7.0 by addition of sodium hydroxide. The fermentations were carried out in a stirred aerated medium (1.2 l.) in 2 l. glass bolthead flasks. The cultures were stirred with paddle stirrers (blade, 4.5 × 2 cm.) rotated at 500 r.p.m. and aerated through a porcelain filter (Aerox) with air at 0.5 vol./vol./min. The inoculum for each culture was a suspension containing c. 10×10^6 spores from a 7-day culture on beer wort agar. Good mycelial growth was established after 48 hr. incubation. The mycelium was pink in colour; microscopical examination showed that the hyphae were rough and pitted and there was abundant sporulation. Foaming was controlled by manual addition of octadecanol (2%) in arachis oil. The trichothecin yield remained practically constant for a further 80 hr. after the maximum concentration had been reached and the pH did not change appreciably during this period.

In vitro activity of trichothecin against fungi pathogenic to man

The growth of twenty-seven representative non-pathogenic Fungi Imperfecti, Zygomycetes and Ascomycetes was inhibited to some degree by trichothecin (Freeman & Morrison, 1949*b*). *In vitro* tests were carried out with

eighteen cultures of pathogenic fungi, including some dermatophytes, in the presence of trichothecin concentrations of 0.64–80 mg./l. Growth of the following fungi was inhibited at 16 mg. trichothecin/l. or less: *Blastomyces dermatitidis*, *Torulopsis neoformans*, *Candida albicans*, *Hormodendrum langeroni*, *Histoplasma capsulatum*, *Epidermophyton floccosum*, *Trichophyton rubrum*, *T. interdigitale*, *T. sulphurum*, *T. mentagrophytes* and *Microsporium canis* (Table 1). Where tests were made, the antifungal activity was not significantly influenced by the presence of blood serum (5%) in the culture medium.

Table 1. In vitro activity of trichothecin on the growth of certain pathogenic fungi

The fungi were seeded on to the surface of agar media containing a series of trichothecin concentrations. After 5 days of incubation at 25° growth was compared with that obtained on control plates containing no antifungal substance. No growth, 0; trace of growth, 1; slight growth, 2; good growth, 3; abundant growth (as control), 4.

Organism	Laboratory catalogue no.	Medium	Trichothecin concentration (mg./l.)			
			80	16	3.2	0.64
			Relative growth			
<i>Actinomyces asteroides</i> (Epp.) Gasp.	278	Nutrient agar + 5% serum	4	4	4	4
<i>Coccidioides immitis</i> Stiles	279	Beer wort agar	4	4	4	4
<i>Blastomyces dermatitidis</i> (Gilchrist and Stokes)	280	Nutrient agar + 5% serum	1	1	1	3
<i>B. dermatitidis</i> (yeast-like form)	291	Nutrient agar + 1% glucose	0	1	2	3
<i>Torulopsis neoformans</i> Lodder	281	Nutrient agar	0	0	2	3
<i>T. neoformans</i> Lodder	281	Nutrient agar + 5% serum	0	0	2	3
<i>Candida albicans</i> (Robin) Berkh.	282	Nutrient agar	0	0	3	4
<i>C. albicans</i> (Robin) Berkh.	282	Nutrient agar + 5% serum	0	0	3	4
<i>Hormodendrum langeroni</i>	283	Beer wort agar	0	1	3	4
<i>Sporotrichum tropicale</i>	286	Nutrient agar + 1% glucose	2	3	4	4
<i>S. schencki</i> Matr.	287	Nutrient agar + 1% glucose	3	4	4	4
<i>S. beurmanni</i> Matr. & Ramond	288	Nutrient agar + 1% glucose	2	3	4	4
<i>Geotrichum cutaneum</i>	289	Nutrient agar + 1% glucose	0	3	4	4
<i>Histoplasma capsulatum</i> Darling	290	Nutrient agar + 1% glucose	0	0	0	3
<i>Epidermophyton floccosum</i> (Harz) Lang. and Miloch.	294	Beer wort agar	0	1	2	3
<i>Trichophyton rubrum</i> Semon	295	Beer wort agar	0	0	4	4
<i>T. interdigitale</i> Priestley	296	Beer wort agar	0	1	4	4
<i>T. sulphureum</i> Sab.	297	Beer wort agar	0	1	2	3
<i>T. mentagrophytes</i> Gedoelst	298	Beer wort agar	0	0	2	4
<i>Microsporium canis</i> Bodin	299	Beer wort agar	0	0	3	3

Toxicity tests in animals

Unfortunately the antifungal activity of trichothecin is of no practical importance in treating infections in animals owing to the high toxicity of the antibiotic. The following tests of toxicity of trichothecin on intravenous injection into mice were carried out by Dr A. R. Martin. Trichothecin (0.25 g.) was dispersed in 2.5 ml. of a solution containing 'Dispersol' OG (5%) and methyl ethyl cellulose (0.1%) by milling overnight with steel balls. Further dilutions of the resulting suspension (100 mg./ml.) were made with the above

solution of dispersing agents. Groups of three mice (18–22 g.) were injected intravenously with 0.1 ml. trichothecin suspension; each injection was made at a steady rate over a period of 15 sec. The results noted in Table 2 were observed. To confirm that the immediate collapse observed after injection of trichothecin was not due to mechanical blockage of capillaries by particles of the antibiotic, suspensions of sulphathiazole, milled under conditions similar to those used for trichothecin, were injected into mice. Milling of the sulphathiazole suspension was arrested when the particles of the drug were of the same order of size as those of the trichothecin suspension. Injection of 10 mg. of sulphathiazole in this form did not cause any immediate collapse comparable with that produced by the injection of 1 mg. trichothecin. Injection of a second trichothecin suspension, which was ball-milled for 40 hr. and contained a smaller proportion of relatively large particles than that described above gave results not significantly different from those of the previous preparation. It was concluded that the observed toxicity was an intrinsic property of the antibiotic, independent of its physical form.

Table 2. *Results of toxicity tests of trichothecin in mice*

Dose of trichothecin		Effect
(mg./20 g. mouse)	(mg./kg.)	
0.1	5	None
0.25	12.5	Transient flaccid paralysis of hind legs lasting c. 20 sec.
0.5	25	Paralysis of hind legs followed by unsteady gait, recovery after 2–5 min.
1.0	50	Collapse immediately after injection. Hind legs paralysed for about 5 min. Animals recovered
2.5	125	Collapse followed by recovery
5.0	250	Initial collapse. Two mice began to recover after 4 min., third mouse died. Normal gait in 2 survivors after 10 min.
10.0	500	Collapse followed by death of all mice within 30 sec.

Three rats (100 g.) were injected subcutaneously with a solution of trichothecin (25 mg.) in arachis oil (0.5 ml.) (dose, 250 mg./kg.). The animals became ill after 1.5 hr. with severe diarrhoea and excessive micturition and were dead after 16 hr. Post-mortem examination confirmed the observation of diarrhoea by the complete absence of formed faeces in the large intestine. The livers were paler than normal.

When trichothecin (5 mg.) as a 1 % solution in arachis oil was applied daily to shaved guinea-pig skin, reddening and soreness followed by cracking and pin-point haemorrhages were observed after five applications. Similar treatment of the inner surface of a rabbit's ear resulted in reddening of the skin followed by scaly patches after three applications. One drop of the 1 % solution applied to the skin of the human head caused definite irritation. Four drops of the same solution (two drops at 2 hr. intervals) introduced into a rabbit's eye caused slight inflammation of the conjunctiva, nictitating mem-

brane and lower eyelid. Gorlenko (1948) reported the presence of endotoxins in the mycelium of *Trichothecium roseum* and other moulds. This toxicity does not appear to be associated with that of trichothecin since the medium upon which the mould was grown was free from the toxin.

Antifungal activity of various esters related to trichothecin

Trichothecin ($C_{19}H_{24}O_5$) is the *isocrotonyl* ester of the ketonic alcohol trichothecolone ($C_{15}H_{20}O_4$; Freeman & Gill, 1950). The following synthetic esters related to trichothecin were prepared: acetyltrichothecolone, acetyldihydrotrichothecolone, crotonyltrichothecolone and butyryltrichothecolone (Table 3). Hydrolysis of trichothecin with boiling aqueous sodium hydroxide gave *isocrotonic* acid and a ketonic alcohol, *isotrichothecolone*, isomeric with trichothecolone (Freeman & Gill, 1950). The corresponding acetyl ester, acetyl *isotrichothecolone*, was prepared and its biological properties examined.

Functional groups in the trichothecolone portion of the molecule include an ethylenic double bond and an endomethylene oxy bridge. The ethylenic double bond is saturated in dihydrotrichothecolone and the corresponding tetrahydrotrichothecin. The endomethylene oxy bridge may be opened with the addition of the elements of water and of hydrogen chloride with formation of trichothecin glycol and trichothecin chlorohydrin. Details of the preparation and properties of these esters and derivatives will be published elsewhere. Their antifungal activities against six test fungi were determined by plate tests in which the substances were dissolved in the beer wort agar (Table 3).

The results showed that acetyltrichothecolone, butyryltrichothecolone and crotonyltrichothecolone were of the same order of activity as trichothecin. Trichothecolone, acetyldihydrotrichothecolone, and tetrahydrotrichothecin exhibited very slight activity against some of the test fungi and none against others which were less sensitive. *Isotrichothecolone*, acetyl*isotrichothecolone*, trichothecin hydrate and trichothecin chlorohydrin were inactive.

The relative activity of these substances was determined more accurately by measurement of their inhibition of germination of *Penicillium digitatum* conidia. The basis of the method was described by the Committee on Standardization of Fungicidal Tests of the American Phytopathological Society (1947). Its use for trichothecin determination was described by Freeman & Morrison (1949*b*); the results are summarized in Table 4. It is concluded that the antifungal activity of trichothecin is a function of the molecule as a whole. Saturation of the ethylenic double bond in the trichothecolone portion of the molecule (as in acetyldihydrotrichothecolone and tetrahydrotrichothecin) destroyed the activity almost completely. Complete destruction of the activity followed isomerization with hot alkali (*isotrichothecolone* and the corresponding ester, acetyl*isotrichothecolone*) or opening of the endomethylene-oxy bridge by addition of the elements of water or of hydrogen chloride (as in trichothecin glycol and trichothecin chlorohydrin, respectively).

Table 4. *Activity of various esters and trichothecin derivatives as inhibitors of germination of Penicillium digitatum conidia*

Germination of *Penicillium digitatum* conidia in Czapek-Dox medium containing 5% (w/v) glucose at pH 4.2 was observed after 18 hr. incubation at 25° in the presence of a series of concentrations of the substances. The concentration which permitted 50% germination (ED 50) was determined graphically.

Substance	ED 50 (mg./l.)	Activity as % that of trichothecin
Trichothecin*	0.38	100
Acetyltrichothecolone†	0.47	81
Crotonyltrichothecolone‡	1.6	23.7
Butyryltrichothecolone‡	1.9	20
Acetyldihydrotrichothecolone	6.6	5.8
Tetrahydrotrichothecin	23	1.7
Trichothecolone	34	1.1
Isotrichothecolone	> 50	< 0.8
Acetylisotrichothecolone	> 50	< 0.8
Trichothecin glycol	> 50	< 0.8
Trichothecin chlorohydrin	> 50	< 0.8

* Germination virtually completely inhibited by 0.78 mg./l.; 100% germination at 0.19 mg./l.

† Germination virtually completely inhibited by 1 mg./l.; 100% germination at 0.26 mg./l.

‡ Germination virtually completely inhibited by 3 mg./l.; 100% germination at 0.77 mg./l.

Antifungal activity of trichothecin in the presence of β -indolylacetic acid

Dr W. G. Templeman has observed that trichothecin inhibits the activity of β -indolylacetic acid and other plant growth regulators (private communication). About two molecular proportions of trichothecin are required for complete inhibition of the activity of one molecule of β -indolylacetic acid. None of the known reactions of trichothecin suggests that combination with β -indolylacetic acid in these proportions does, in fact, occur. As shown above, all the known modifications to the trichothecin molecule, with the exception of substitution of other acidic groups for the *isocrotonyl* radical in the ester, result in complete or almost complete destruction of the antifungal activity. Incubation of as much as 60 equivalents of β -indolylacetic acid with trichothecin had no effect on the germination of *P. digitatum* conidia in presence of the mixture, as compared with conidia incubated with the corresponding quantities of trichothecin (Table 5). This evidence suggests that no interaction takes place between β -indolylacetic acid and trichothecin in aqueous solution, under these conditions, since it is regarded as unlikely that combination would take place without affecting the antifungal activity.

DISCUSSION

The work reported above on the biological activity of trichothecin and its derivatives has not revealed any close relationship between the functional groups in the molecule and its antifungal activity. The evidence so far as it goes indicates that the activity is dependent upon the molecular structure as a whole, since all the modifications which it has been possible to introduce,

with the exception of substitution of the *isocrotonyl* radical by other acidic groups in the ester, have resulted in complete or almost complete destruction of the activity.

Table 5. *Germination of conidia of Penicillium digitatum in the presence of mixtures of trichothecin and β -indolylacetic acid*

The experimental conditions were similar to those described in Table 4. Solutions containing twice the concentrations of trichothecin and β -indolylacetic acid, shown below, were incubated at 25° for 1.5 hr. before addition of equal volumes of the spore suspension.

There was 100 % germination in presence of β -indolylacetic acid (up to 50 mg./l.) alone.

β -indolylacetic acid (mg./l.)	Germination of conidia in presence of trichothecin (mg./l.)					ED 50 (mg./l.)
	1.25	0.625	0.313	0.156	0.078	
	% germination					
Nil	0	50	80	100	100	0.50
0.025	0	60	80	100	100	0.52
0.25	0	40	80	100	100	0.48
2.5	0	60	80	100	100	0.52
25	1	50	80	100	100	0.52

Data are now beginning to accumulate on the action of trichothecin, and in some cases also of its derivatives, towards a number of widely different biological systems including fungi, viruses, flowering plants and mammals. Different molecular functions are probably responsible for the activity in certain of these fields. Thus, whereas the antifungal activity of trichothecin is practically lost in its derivatives, with the exception of certain esters, trichothecolone and acetyltrichothecolone more effectively prevented infection of *Nicotiana glutinosa* by two test viruses than did trichothecin (Bawden & Freeman, 1952). Similar comparisons of the activities of trichothecin and its derivatives towards plant growth regulators and comparisons of their toxicities in animals may lead to interesting conclusions.

I wish to express my thanks to Mr A. J. Baillie for his assistance with the anti-fungal assays.

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Logarithms to Base 2

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Graphical representation of the growth of a bacterial culture is usually best achieved by using a logarithmic scale for whatever measurement of the density of the culture is made. Moreover, as Monod (1949) has pointed out, both diagrams and associated calculations are more readily interpreted if logarithms to base 2 are used instead of common logarithms (i.e. base 10): an increase of 1 unit on the logarithmic scale then corresponds to the equivalent of one division by each of the cells present at the beginning of the interval.

Rather surprisingly, none of the better-known collections of mathematical tables nor any of the usual sources for miscellaneous tables appears to contain logarithms to base 2. Of course, for any particular number, multiplication of its common logarithm by 3.32193 ($=\log_2 10$) gives the logarithm to base 2, but performance of this multiplication for many series of data is tedious and time consuming. As the result of a suggestion from Dr W. E. van Heyningen, the following table has therefore been computed.† Only 3 places of decimals are shown, since graphical work will certainly not permit greater accuracy and even for arithmetical analysis observational data rarely justify the use of more digits; the table is designed for simple rapid use by microbiologists, not for serious mathematical purposes.

The table shows the logarithm of every integer from 100 to 999. Thus, by direct reading,

$$\log_2 673 = 9.394.$$

If the highest and lowest densities of a series do not differ by more than a factor of 10, there may be no necessity to construct any logarithms outside the range of the table. For example, if the densities range from 1.7 to 8.9, expression of density in terms of a volume unit 100 times that of the standard will give values ranging from 170 to 890; all logarithms can then be read directly from the table and graphed as they stand.

If the densities extend over a wider range, logarithms of numbers not in the table will be needed. These are obtained by the usual rule that the logarithm of a product or quotient is the sum or difference of the logarithms of the two parts. Thus

$$\begin{aligned}\log_2 2692 &= \log_2 4 + \log_2 673 \\ &= 2.000 + 9.394 = 11.394, \\ \log_2 0.075 &= \log_2 300 - \log_2 400 - \log_2 10 \\ &= 8.229 - 8.644 - 3.322 = -3.737.\end{aligned}$$

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† The table was obtained by multiplication of appropriate entries in the *U.S. National Bureau of Standards Table of Natural Logarithms* by $\log_2 e$ and independently checked by multiplication of entries in *Chamber's Mathematical Tables* (Seven Figure Logarithms) by $\log_2 10$.

When suitable factors are not obvious, powers of 10 can be used, remembering that $\log_2 10 = 3.3219$, $\log_2 100 = 6.6439$, $\log_2 1000 = 9.9658$; logarithms of powers of 10 up to 10^{10} are appended to the table. Hence

$$\begin{aligned}\log_2 0.875 &= \log_2 875 - \log_2 1000 \\ &= 9.773 - 9.966 = -0.193.\end{aligned}$$

(Since $0.875 = 7/8$, the same value can be obtained as $\log_2 700 - \log_2 800$.) The measurements will rarely be accurate to more than 3 significant digits, but, if required, logarithms of quantities intermediate between tabular values can be derived by the common-sense application of 'linear interpolation'; thus $\log_2 2692$ can alternatively be calculated as at 2/10 of the distance between

$$\log_2 2690 = 8.071 + 3.322 = 11.393,$$

and

$$\log_2 2700 = 8.077 + 3.322 = 11.399,$$

and is 11.394 as before.

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Logarithms to base 2

	0	1	2	3	4	5	6	7	8	9
100	6·644	6·658	6·672	6·687	6·700	6·714	6·728	6·741	6·755	6·768
110	·781	·794	·807	·820	·833	·845	·858	·870	·883	·895
120	·907	·919	·931	·943	·954	·966	·977	·989	7·000	7·011
130	7·022	7·033	7·044	7·055	7·066	7·077	7·087	7·098	·109	·119
140	·129	·140	·150	·160	·170	·180	·190	·200	·209	·219
150	7·229	7·238	7·248	7·257	7·267	7·276	7·285	7·295	7·304	7·313
160	·322	·331	·340	·349	·358	·366	·375	·384	·392	·401
170	·409	·418	·426	·435	·443	·451	·459	·468	·476	·484
180	·492	·500	·508	·516	·524	·531	·539	·547	·555	·562
190	·570	·577	·585	·592	·600	·607	·615	·622	·629	·637
200	7·644	7·651	7·658	7·665	7·672	7·679	7·687	7·693	7·700	7·707
210	·714	·721	·728	·735	·741	·748	·755	·762	·768	·775
220	·781	·788	·794	·801	·807	·814	·820	·827	·833	·839
230	·845	·852	·858	·864	·870	·877	·883	·889	·895	·901
240	·907	·913	·919	·925	·931	·937	·943	·948	·954	·960
250	7·966	7·972	7·977	7·983	7·989	7·994	8·000	8·006	8·011	8·017
260	8·022	8·028	8·033	8·039	8·044	8·050	·055	·061	·066	·071
270	·077	·082	·087	·093	·098	·103	·109	·114	·119	·124
280	·129	·134	·140	·145	·150	·155	·160	·165	·170	·175
290	·180	·185	·190	·195	·200	·205	·209	·214	·219	·224
300	8·229	8·234	8·238	8·243	8·248	8·253	8·257	8·262	8·267	8·271
310	·276	·281	·285	·290	·295	·299	·304	·308	·313	·317
320	·322	·326	·331	·335	·340	·344	·349	·353	·358	·362
330	·366	·371	·375	·379	·384	·388	·392	·397	·401	·405
340	·409	·414	·418	·422	·426	·430	·435	·439	·443	·447
350	8·451	8·455	8·459	8·464	8·468	8·472	8·476	8·480	8·484	8·488
360	·492	·496	·500	·504	·508	·512	·516	·520	·524	·527
370	·531	·535	·539	·543	·547	·551	·555	·558	·562	·566
380	·570	·574	·577	·581	·585	·589	·592	·596	·600	·604
390	·607	·611	·615	·618	·622	·626	·629	·633	·637	·640
400	8·644	8·647	8·651	8·655	8·658	8·662	8·665	8·669	8·672	8·676
410	·679	·683	·687	·690	·693	·697	·700	·704	·707	·711
420	·714	·718	·721	·725	·728	·731	·735	·738	·741	·745
430	·748	·752	·755	·758	·762	·765	·768	·771	·775	·778
440	·781	·785	·788	·791	·794	·798	·801	·804	·807	·811
450	8·814	8·817	8·820	8·823	8·827	8·830	8·833	8·836	8·839	8·842
460	·845	·849	·852	·855	·858	·861	·864	·867	·870	·873
470	·877	·880	·883	·886	·889	·892	·895	·898	·901	·904
480	·907	·910	·913	·916	·919	·922	·925	·928	·931	·934
490	·937	·940	·943	·945	·948	·951	·954	·957	·960	·963
500	8·966	8·969	8·972	8·974	8·977	8·980	8·983	8·986	8·989	8·992
510	·994	·997	9·000	9·003	9·006	9·008	9·011	9·014	9·017	9·020
520	9·022	9·025	·028	·031	·033	·036	·039	·042	·044	·047
530	·050	·053	·055	·058	·061	·063	·066	·069	·071	·074
540	·077	·079	·082	·085	·087	·090	·093	·085	·098	·101

Logarithms to base 2

	0	1	2	3	4	5	6	7	8	9
550	9·103	9·106	9·109	9·111	9·114	9·116	9·119	9·122	9·124	9·127
560	·129	·132	·134	·137	·140	·142	·145	·147	·150	·152
570	·155	·157	·160	·162	·165	·167	·170	·172	·175	·177
580	·180	·182	·185	·187	·190	·192	·195	·197	·200	·202
590	·205	·207	·210	·212	·214	·217	·219	·222	·224	·226
600	9·229	9·231	9·234	9·236	9·238	9·241	9·243	9·246	9·248	9·250
610	·253	·255	·257	·260	·262	·264	·267	·269	·271	·274
620	·276	·278	·281	·283	·285	·288	·290	·292	·295	·297
630	·299	·301	·304	·306	·308	·311	·313	·315	·317	·320
640	·322	·324	·326	·329	·331	·333	·335	·338	·340	·342
650	9·344	9·347	9·349	9·351	9·353	9·355	9·358	9·360	9·362	9·364
660	·366	·369	·371	·373	·375	·377	·379	·382	·384	·386
670	·388	·390	·392	·394	·397	·399	·401	·403	·405	·407
680	·409	·412	·414	·416	·418	·420	·422	·424	·426	·428
690	·430	·433	·435	·437	·439	·441	·443	·445	·447	·449
700	9·451	9·453	9·455	9·457	9·459	9·461	9·464	9·466	9·468	9·470
710	·472	·474	·476	·478	·480	·482	·484	·486	·488	·490
720	·492	·494	·496	·498	·500	·502	·504	·506	·508	·510
730	·512	·514	·516	·518	·520	·522	·524	·526	·527	·529
740	·531	·533	·535	·537	·539	·541	·543	·545	·547	·549
750	9·551	9·553	9·555	9·557	9·558	9·560	9·562	9·564	9·566	9·568
760	·570	·572	·574	·576	·577	·579	·581	·583	·585	·587
770	·589	·591	·592	·594	·596	·598	·600	·602	·604	·605
780	·607	·609	·611	·613	·615	·617	·618	·620	·622	·624
790	·626	·628	·629	·631	·633	·635	·637	·638	·640	·642
800	9·644	9·646	9·647	9·649	9·651	9·653	9·655	9·656	9·658	9·660
810	·662	·664	·665	·667	·669	·671	·672	·674	·676	·678
820	·679	·681	·683	·685	·687	·688	·690	·692	·693	·695
830	·697	·699	·700	·702	·704	·706	·707	·709	·711	·713
840	·714	·716	·718	·719	·721	·723	·725	·726	·728	·730
850	9·731	9·733	9·735	9·736	9·738	9·740	9·741	9·743	9·745	9·747
860	·748	·750	·752	·753	·755	·757	·758	·760	·762	·763
870	·765	·767	·768	·770	·771	·773	·775	·776	·778	·780
880	·781	·783	·785	·786	·788	·790	·791	·793	·794	·796
890	·798	·799	·801	·803	·804	·806	·807	·809	·811	·812
900	9·814	9·815	9·817	9·819	9·820	9·822	9·823	9·825	9·827	9·828
910	·830	·831	·833	·834	·836	·838	·839	·841	·842	·844
920	·845	·847	·849	·850	·852	·853	·855	·856	·858	·860
930	·861	·863	·864	·866	·867	·869	·870	·872	·873	·875
940	·877	·878	·880	·881	·883	·884	·886	·887	·889	·890
950	9·892	9·893	9·895	9·896	9·898	9·899	9·901	9·902	9·904	9·905
960	·907	·908	·910	·911	·913	·914	·916	·917	·919	·920
970	·922	·923	·925	·926	·928	·929	·931	·932	·934	·935
980	·937	·938	·940	·941	·943	·944	·945	·947	·948	·950
990	·951	·953	·954	·956	·957	·959	·960	·961	·963	·964
	10	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ⁷	10 ⁸	10 ⁹	10 ¹⁰
	3·322	6·644	9·966	13·288	16·610	19·932	23·253	26·575	29·897	33·219

A Fluorescent Derivative of Polymyxin: its Preparation and Use in Studying the Site of Action of the Antibiotic

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SUMMARY: A fluorescent derivative of polymyxin (DANSP) was prepared by coupling 1-dimethylaminonaphthalene-5-sulphonyl chloride with the γ amino group of $\alpha\gamma$ diaminobutyric acid radicals in the polymyxin molecule. As in the case of polymyxin, DANSP is readily absorbed by polymyxin-sensitive organisms and is rapidly bactericidal; cells can be protected against this bactericidal activity by pretreatment with certain cations. Mechanical disintegration of DANSP-treated cells showed that the fluorescent compound was associated with two fractions: (a) cell walls and (b) small particles. Fractionation of three strains of *Pseudomonas aeruginosa* showed that the fluorescent conjugate was equally distributed between the two fractions, but in the case of three Gram-positive, lysozyme-sensitive, organisms 90 % of the fluorescent conjugate was found in the small particle fraction; controlled lysozyme treatment of these cells showed that the DANSP was associated with a membrane underlying the cell wall from which the small particle fraction is formed on mechanical disintegration.

Previous studies (Few & Schulman, 1953; Newton, 1953*a*) have shown that the addition of polymyxin to washed cell suspensions of sensitive organisms causes a release of soluble constituents from the cells; it was suggested by these workers that the bactericidal activity of polymyxin might be due to its ability to combine with certain groups near the cell surface and, in so doing, cause a disorganization of a cell membrane or osmotic barrier. Sensitive organisms can be protected against the bactericidal activity of polymyxin by certain cations (Newton, 1953*b*). This protection is due to a competition between polymyxin and cations for sites on the cells, pretreatment of the cells with cations preventing absorption of the antibiotic. A comparison of the affinities of a number of bi- and trivalent cations for the polymyxin-combining groups of a strain of *Pseudomonas aeruginosa* with the ability of these cations to reverse the charge on certain types of colloids suggested that the polymyxin-combining loci of these cells may be polyphosphates (Newton, 1954*a*).

As yet there has been no account of the localization of polymyxin within the bacterial cell; in a preliminary communication (Newton, 1954*b*) it was reported that cell walls prepared from a polymyxin-sensitive strain of *Pseudomonas aeruginosa* by mechanical disintegration rapidly absorbed as much as 300 μg . polymyxin/mg. dry wt. cell walls, whereas cell walls prepared from a polymyxin-resistant strain of the same organism absorbed less than 70 μg ./mg.; electron microscopy showed that cell walls of sensitive strains were more electron-dense after polymyxin treatment. Similar results have been obtained with cell wall preparations from a number of other organisms

(Few, 1954), and Few & Schulman (1953) state that 'the isotherms for the absorption of polymyxin by both intact bacteria and bacterial cell wall preparations favour absorption at sites within the bacterial cell wall with but little penetration inside the cell'. However, experiments with isolated cell walls are difficult to interpret and give no indication of the amount of antibiotic which would be absorbed by the cell wall when other cellular components are present. A more direct approach to this problem has been made possible by the preparation of a fluorescent derivative of polymyxin.

Weber (1952) described the preparation of fluorescent conjugates of ovalbumin and bovine serum albumin with 1-dimethylaminonaphthalene-5-sulphonyl chloride. He found that coupling with the dye did not induce any observable denaturation of ovalbumin as judged by solubility at the isoelectric point; also that the enzymic properties of fumarase and ribonuclease were unaffected by coupling 1 to 3 molecules of the naphthalene derivative per protein molecule. There is a large difference in affinity of sulphonyl chloride for —OH and =NH compared with —NH_2 groups and it seems likely that only $\text{—SO}_2\text{NH—}$ bonds are formed, although this has not yet been demonstrated. Weber found no evidence for spontaneous breakdown of the conjugates, as shown by the appearance of free naphthalene sulphonic acid, while the fluorescence of the compounds was unaffected by changes of pH between 1.6 and 14.

Polymyxin B is a basic peptide of molecular weight *c.* 1200; it contains the amino acids phenylalanine, D-leucine, L-threonine and L- $\alpha\gamma$ -diaminobutyric acid in addition to a C_9 saturated fatty acid identified as methyl octan-1-oic acid (Wilkinson, 1949). A cyclic structure has been postulated for the molecule by Bell *et al.* (1949) who identified the free amino groups of intact polymyxin with the γ -amino groups of $\alpha\gamma$ -diaminobutyric acid and found five molecules of this amino acid per polymyxin molecule. A fluorescent derivative of polymyxin has been prepared by coupling 1-dimethylaminonaphthalene-5-sulphonyl chloride with the free amino groups of the antibiotic; limiting amounts of the sulphonyl compound being used so that each polymyxin molecule carried on the average one naphthalene group. The derivative (DANSP in the sequel) had approximately the same bactericidal activity as untreated polymyxin. The present paper describes the preparation of the compound and its localization in polymyxin sensitive organisms.

METHODS

Organisms. Organisms used in this work were three strains of *Pseudomonas aeruginosa* isolated by Dr E. J. L. Lowbury at the Birmingham Accident Hospital; *Bacillus megaterium* strain KM; a laboratory strain of *Sarcina lutea* and one of *Micrococcus lysodeikticus* (NCTC no. 2665).

Media, conditions of growth and harvesting. Strains of *Pseudomonas aeruginosa* were grown in tryptic digest of casein containing the equivalent of 3% (w/v) casein at an initial pH value of 7.4–7.6, the conditions of culture and harvesting being the same as previously described (Newton, 1953*a*).

Bacillus megaterium, *Sarcina lutea* and *Micrococcus lysodeikticus* were grown in 2% Difco peptone contained in Pyrex tubes (4×20 cm.) with a maximal depth of liquid of 8 cm. Tubes were inoculated with 2 ml. of an overnight culture in the same medium and the organisms grown for 15 hr. at 30° with aeration. Cells were harvested by centrifugation, washed once in 0.03 M-phosphate buffer (pH 7.0) and resuspended in the same buffer to give a suspension of *c.* 10 mg. dry wt. cells/ml. Dry weights were determined on a Hilger absorptiometer previously calibrated against the organisms used.

Preparation of 1-dimethylaminonaphthalene-5-sulphonamidopolymyxin (DANSP). Polymyxin B sulphate (250 mg.) and sodium bicarbonate (50 mg.) were dissolved in water, cooled to 2° and mixed with a solution of 1-dimethylaminonaphthalene-5-sulphonyl chloride (60 mg.) in 0.5 ml. acetone; the mixture was shaken for 3 hr. and the resulting conjugate separated from any free sulphonate by repeated precipitation with ethanol followed by slow filtration through a column of Dowex 2 (mesh 200, alkaline form). This treatment removed all traces of the free acid, small amounts of which were readily detected by paper chromatography as below.

Chromatographic separation of DANSP and 1-dimethylaminonaphthalene-5-sulphonic acid. The materials dissolved in 60% ethanol were spotted on Whatman no. 1 filter-paper, molar phosphate buffer, pH 7.0 was used as solvent and ascending chromatograms were run for 45 min. Papers were photographed in ultraviolet light using a light filter of saturated sodium nitrite solution 1 cm. thick (Weber, 1953). The R_F value of the sulphonamide derivative was effectively 0 and that of the sulphonate was 0.6; Pl. 1, fig. 1, is a photograph of a chromatogram showing the removal of free sulphonate by filtration through Dowex 2.

Absorption spectra of 1-dimethylaminonaphthalene-5-sulphonic acid, polymyxin B and DANSP. The absorption spectra of 1-dimethylaminonaphthalene-5-sulphonic acid, polymyxin B and DANSP are shown in Fig. 1. Polymyxin B shows an absorption maximum at $256 m\mu$. due to its phenylalanine content. The sulphonic acid and its sulphonamide derivatives show an absorption band in the 300–400 $m\mu$. region; the position of the maximum depends upon the ionic character of the $=SO_2$ group (Weber, 1952); coupling with polymyxin resulted in a significant displacement towards the red (from 315 to 333 $m\mu$.).

Determination of limiting inhibitory concentrations of polymyxin B and DANSP for Pseudomonas aeruginosa and Bacillus megaterium. Serial dilutions of polymyxin B sulphate or DANSP were made into sterile tryptic digest of casein contained in test tubes (2.5×15 cm.) which were closed with 'Oxoid' metal caps. Tubes, containing 5 ml., were inoculated with *c.* 10^5 viable cells/ml. medium and incubated at 30° for 24 hr. The concentration of antibiotic in the first tube which showed no growth after 24 hr. was taken as the limiting inhibitory concentration; incubation for 48 hr. did not alter the end point.

Plate-count estimation of surviving cells. Bacterial suspensions were treated with different concentrations of the antibiotic for 5 min. at 30° , 1 ml. samples

were then withdrawn and transferred to 99 ml. sterile saline (1 %, w/v), suitable dilutions in saline were then plated out in casein digest growth medium containing 2 % agar and the colonies counted after 48 hr. incubation at 30°.

Mechanical disintegration of bacteria and isolation of cell walls. The procedure used was essentially the same as that described by Salton (1953). Ten ml. of a washed cell suspension containing *c.* 10 mg. dry wt. cells/ml. were shaken with 10 ml. ballotini glass beads (grade 12), in the Mickle disintegrator (Mickle, 1948) for 15 min. The ballotini were removed by filtration through

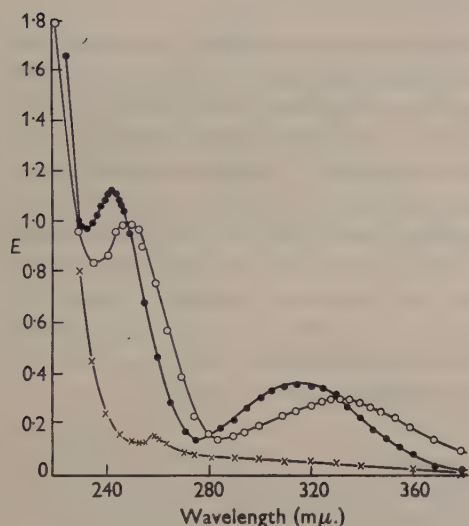


Fig. 1.

Fig. 1. *Absorption spectra.* ●—●, 1-dimethylaminonaphthalene-5-sulphonic acid (100 $\mu\text{g./ml.}$ in 60 % ethanol); ×—×, polymyxin B sulphate (500 $\mu\text{g./ml.}$ in 60 % ethanol); ○—○, 1-dimethylaminonaphthalene-5-sulphonamido-polymyxin (DANSP; 100 $\mu\text{g./ml.}$ in 60 % ethanol).

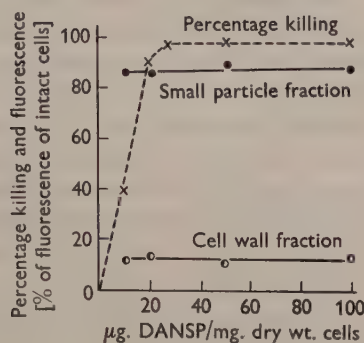


Fig. 2.

Fig. 2. *Distribution of DANSP between the cell wall and small particle fractions of disrupted *Bacillus megaterium*.* Washed cells of *B. megaterium* treated with various concentrations of DANSP for 20 min. at 30° and washed repeatedly in distilled water until no fluorescence remained in washings. Intensity of fluorescence of intact cells and of cell fractions obtained by mechanical disintegration and differential centrifugation. Fluorescence of cell fractions expressed as percentage of fluorescence of intact cells. Number of cells surviving DANSP treatment estimated by plate counts.

a no. 1 sintered glass funnel. Under these conditions of disintegration more than 90 % of the cells were broken; any intact cells were removed from suspension by centrifugation for 10 min. at 1000 *g*. The cell walls were then separated from the 'cytoplasmic fraction' by centrifugation for 20 min. at 10,000 *g*, washed once in 1 *M*-sodium chloride and then repeatedly with distilled water until the supernatant fluids no longer gave a precipitate with silver nitrate. Small particles were separated from the 'cytoplasmic fraction' by centrifugation for 15 min. at 100,000 *g* in a Spinco Model L centrifuge.

Lysozyme treatment of Bacillus megaterium. Protoplasts were prepared from *B. megaterium* by treatment with lysozyme under the conditions described by Weibull (1953).

Fluorescence microscopy. Fluorescence photomicrographs were taken by Mr J. Smiles and Mr M. R. Young of the National Institute for Medical Research, Mill Hill, London, using a high-pressure mercury vapour lamp (Type G.E.C. 'Osira' 80 W) as ultraviolet light source with a Chance 0 × 7 filter and 4% nickel sulphate solution as primary filter. The secondary filter placed in the projection ocular was a Leitz 'blue-green' filter. Photographic plates were Ilford Selochrome; exposure time *c.* 10 min.

Measurement of intensity and polarization of fluorescence. Intensities of fluorescence of DANSP-treated cell suspensions and cell fractions were measured by comparison with a standard fluorescent solution using a modified Pulfrich photometer as previously described (Newton, 1954*a*). The polarization of fluorescence was determined by the method of Weber (1952).

Preparation of specimens for the electron microscope. Specimens were washed twice with distilled water and finally suspended in distilled water. Protoplasts and protoplast membranes were fixed with mercuric chloride-formalin solution before washing. Micro-drops of suspensions were placed on specimen grids covered with nitrocellulose film and shadowed with gold palladium alloy (60:40%). The shadowing was at an angle of 30° from the plane of the supporting film. Observations were made in the Siemens electron microscope, generally at a direct magnification of × 10,000–14,000.

RESULTS

Limiting inhibitory concentrations of polymyxin B and DANSP

Growth tests showed that the antibiotic properties of polymyxin were not significantly affected by coupling with 1-dimethylaminonaphthalene-5-sulphonyl chloride. The limiting inhibitory concentration of polymyxin B for *Pseudomonas aeruginosa* was 1.5 µg./ml. in growth tests using an inoculum of *c.* 10⁶ viable cells/ml. In similar growth tests the limiting inhibitory concentration of DANSP was 3.0 µg./ml. Growth of *Bacillus megaterium* was inhibited by 0.75 µg. polymyxin/ml. and by 1.0 µg. DANSP/ml.

Absorption of DANSP by washed cells of Pseudomonas aeruginosa and Bacillus megaterium

Suspensions of washed cells in 1% (w/v) saline (containing *c.* 1 mg. dry wt. cells/ml.) were treated with 25 µg. DANSP/ml. for 5 min. at 30°. After centrifugation the cell pellet and supernatant fluid were examined in ultraviolet light. It was found that the cells had absorbed the DANSP and fluoresced strongly, and that the fluorescence was unaffected by repeated washing with 1% saline. Pl. 1, figs. 2, 3, are fluorescence photomicrographs of *P. aeruginosa* and *B. megaterium* treated with DANSP as described above.

It has previously been shown (Newton, 1953*a,b*) that washed cells of *Pseudomonas aeruginosa* can be protected against the bactericidal activity

of polymyxin by pretreatment with certain cations, the cations preventing the absorption of antibiotic by the cells. Cations also prevent the absorption of DANSP by bacteria. Washed cells of *P. aeruginosa* suspended in 1% (w/v) saline or 1% saline + 0.05 M-calcium chloride were treated with DANSP (25 µg./ml.) for 5 min., the suspensions centrifuged and the cell pellets examined under ultraviolet light. Pl. 1, fig. 4 shows that in the presence of calcium ions little DANSP was absorbed by the cells.

Fractionation of DANSP-treated cells

Experiments with Pseudomonas aeruginosa. Washed cells of *P. aeruginosa* suspended in 1% (w/v) saline were treated with DANSP (25 µg./mg. dry wt. cells) for 20 min. at 30°, the suspension centrifuged, and the cells washed repeatedly with 1% saline until no trace of fluorescence remained in the washings when these were examined under ultraviolet light. The cells were resuspended in 0.01 M-phosphate buffer (pH 6.3) to give a suspension of c. 10 mg. dry wt. cells/ml. and shaken with ballotini in the Mickle disintegrator for 20 min. The cell wall fraction was then isolated as described above. It was found that after removal of the cell walls the supernatant fluid still fluoresced strongly when examined under ultraviolet light; measurement of the intensity of fluorescence of unbroken cells, the cell wall fraction and the 'cytoplasmic fraction' in the Pulfrich photometer showed that the fluorescence was equally distributed between the cell-wall fraction and the 'cytoplasmic fraction'. When the cell walls were shaken with ballotini in the Mickle disintegrator for a further 30 min. centrifugation showed that no fluorescent material had been removed from the walls by this treatment.

The fluorescence of the 'cytoplasmic fraction' was found to be strongly polarized; the degree of polarization did not vary over the temperature range 2–30°, indicating that DANSP was absorbed on to a macromolecule of molecular weight >300,000 (Weber, 1952). Centrifugation of the cytoplasmic fraction at 10,000g for 1 hr. sedimented part of the fluorescent material, the remainder being sedimented after centrifugation for 15 min. at 100,000g. The 'pellet' obtained by this high-speed centrifugation was semi-transparent and dark red in colour; examination of this material in the electron microscope showed that it was mainly composed of small spherical particles 10–30 mµ. in diameter. Examination of a suspension of this small particle fraction with a Zeiss spectroscope showed general absorption below 450 mµ. but no marked bands at longer wavelengths. Addition of sodium dithionite resulted in the immediate appearance of an intense band at 550 mµ. and a weaker band at 559 mµ., suggesting that cytochromes *b* and *c* were associated with the particles. In this respect these particles resemble those isolated from sonic disintegrates of *Pseudomonas fluorescens* by Stanier, Gunsalus & Gunsalus (1953). A similar distribution of DANSP was found when two other strains of *P. aeruginosa* were studied.

Mechanical disintegration of Bacillus megaterium. Washed cells of *B. megaterium* were treated with a range of concentrations of DANSP (10–100 µg.

DANSP/mg. dry wt. cells) for 20 min. at 30°, washed repeatedly with distilled water until no trace of fluorescence remained in the washings, and finally resuspended in distilled water to give a suspension of *c.* 10 mg. dry wt. cells/ml. A sample of this suspension was taken for measurement of fluorescence intensity, the remainder shaken with ballotini in the Mickle disintegrator, and the cell wall and small particle fractions of the disintegrated cells separated by differential centrifugation. After sedimentation of the small particles no fluorescent material remained in the supernatant fluids. The intensity of fluorescence of the cell wall and small particle fractions was measured and expressed as a percentage of the fluorescence of the whole cells. Fig. 2 shows that for a range of four concentrations of DANSP between 10 and 100 µg./mg. dry wt. cells, 90 % of the fluorescence was associated with the small particle fraction and only 10 % with the cell walls. Fig. 2 also shows the relationship between the percentage cells killed and DANSP concentration; *c.* 25 µg. DANSP/mg. dry wt. cells was required for 99 % killing.

When cell walls and small particles were prepared from washed cells of *Bacillus megaterium* it was found that the cell walls alone, on incubation with DANSP, absorbed up to 270 µg. DANSP/mg. dry wt. cells, a figure in close agreement with the values already recorded for the absorption of polymyxin by cell walls of other sensitive organisms (Few & Schulman, 1953; Newton, 1954*b*). The DANSP absorbed by these cell walls was not removed by repeated washing with distilled water. When, however, the DANSP-treated cell walls were incubated with the small particle fraction for 10 min. at 30° and again separated by centrifugation, it was found that more than 90 % of the DANSP had been removed from the cell walls and had been taken up by this small particle fraction.

Lysozyme treatment of Bacillus megaterium. Weibull (1953) showed that when washed cells of *B. megaterium* suspended in dilute phosphate buffer were treated with lysozyme a rapid lysis occurred which left only spherical empty ghosts and small granules. When the cells are suspended in 0.2 M-sucrose during the lysozyme treatment, the bacterial cell wall becomes depolymerized but the rest of the cell remains as an intact structural unit—a spherical 'protoplast' (Pl. 2, fig. 7). Such protoplasts can be lysed by dilution of the sucrose; they then give rise to the ghosts and granules obtained by lysozyme treatment in phosphate buffer. Centrifugation of the lysed protoplasts at 10,000 *g* for 15 min. sediments a dark yellow layer consisting mainly of ghosts, which in the electron microscope appear to be flat membrane-like bodies (Pl. 2, fig. 8). When this fraction is treated for a short period in a sonic oscillator the membrane-like structure is destroyed, but the yellow material can be re-sedimented as a small particle fraction by centrifugation at 100,000 *g*. Weibull found that the cytochromes of the cell are associated with this yellow fraction.

Fluorescence photomicrographs of DANSP-treated *Bacillus megaterium* (Pl. 1, fig. 3) showed that the fluorescent compound was associated with the boundaries of the cell and the cross-walls. When such fluorescent cells were suspended in sucrose solution (0.2 M) and treated with lysozyme, the cell wall

depolymerized, leaving protoplasts which fluoresced strongly in ultraviolet light (Pl. 1, fig. 5). No detectable fluorescent material remained in the supernatant fluids after centrifugation of the protoplast suspension.

When a suspension of fluorescent protoplasts was subjected to supersonic vibration of 25 kc./sec. (generated by a 500 W. Mullard magnetostrictor oscillator) the protoplast structure was completely destroyed. The fluorescent material in the supersonic disintegrate was then found to be associated with a small particle fraction sedimented by centrifugation at 100,000 *g* for 15 min. Similar results were obtained with *Sarcina lutea* and *Micrococcus lysodeikticus*.

*Properties of protoplasts prepared from untreated and
polymyxin treated Bacillus megaterium*

Protoplasts prepared from washed cells of *B. megaterium* which had been treated with a bactericidal concentration of polymyxin or DANSP differed from protoplasts obtained from untreated cells of this organism in that they were not lysed by suspension in distilled water. Further, they consisted of cubical rather than spherical subcellular units, this is clearly shown in the electron micrographs (Pl. 2, figs. 6-9) and was confirmed by phase-contrast microscopy.

DISCUSSION

There is now considerable indirect evidence (Few & Schulman, 1953; Newton, 1953*a*, 1954*a*) that polymyxin combines with and disorganizes structures within the bacterial cell wall which are responsible for the maintenance of the osmotic equilibrium of the cell. A more direct approach to the problem of the site of action of polymyxin has been made possible by studies on protoplasts by the use of a fluorescent derivative of the antibiotic (DANSP). In the experiments described here it has been possible to demonstrate the combination of DANSP with cell walls and small particles from disrupted bacteria, while in the case of certain lysozyme-sensitive organisms it has been found that DANSP combines with a membrane underlying the cell wall. The association of DANSP with the small particles obtained by fractionation of disrupted bacteria is interesting in view of the observations of Burdon (1946) and of Mitchell & Moyle (1951). Burdon described a lipid layer in a number of bacteria which was readily stained by Sudan Black; this lipid layer, which lies inside the cell wall, is usually thought to represent the osmotic barrier of the cell. Mitchell & Moyle (1951) obtained a small particle fraction from *Staphylococcus aureus*; the particles had diameters ranging from 10 to 50 *mμ*. and contained a high proportion of phospholipid. Mitchell & Moyle suggested that the small particles may be associated with the protein envelope in the intact cell as an underlying membrane, and that the lipid of the particles may be the lipid observed by Burdon in bacteria stained with Sudan Black. The more recent work of Weibull (1953) showed that similar particles are formed by sonic disintegration of protoplast membranes which in the intact cell appear to be immediately underlying the cell wall. Thus it seems possible that the small particles which have been shown in the present work to bind the

fluorescent derivative of polymyxin may originate from a membrane which underlies the cell wall.

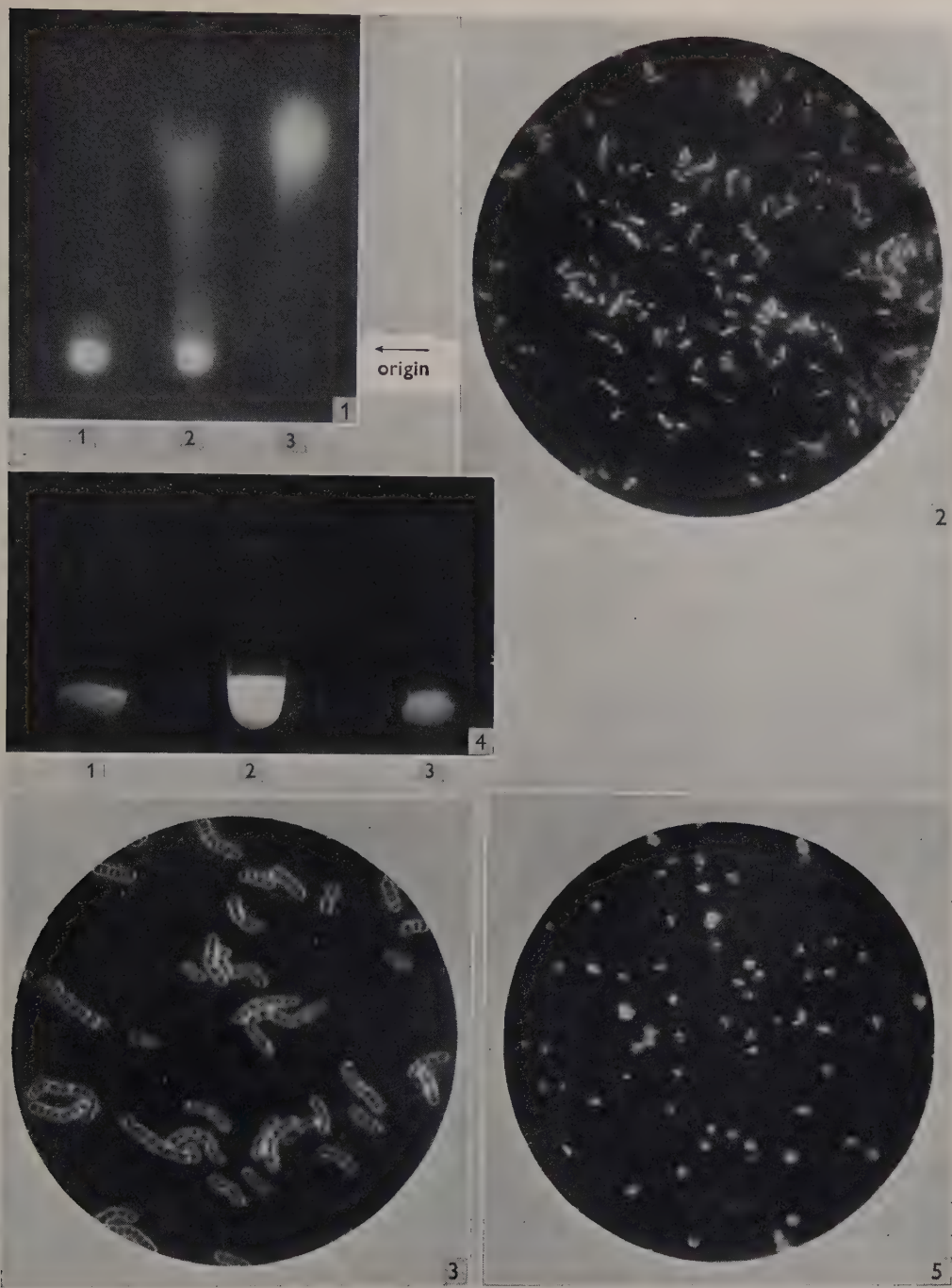
The chemical nature of the polymyxin-binding component of bacterial cells is as yet unknown, but there is some evidence to suggest that it may be a phospholipid. Bliss, Chandler & Schoenbach (1949) showed that phosphatides of the lecithin type interfered with the antibacterial activity of polymyxin; Latterade & Macheboeuf (1950) described the formation of polymyxin-phospholipid complexes; and Newton (1954*a*) showed that the polymyxin-combining groups of *Pseudomonas aeruginosa* had properties of polyphosphates. With this possibility in mind the difference in distribution of DANSP between cell walls and small particles in the case of the two groups of organisms studied might perhaps be explained in terms of the difference in phospholipid content of Gram-positive and Gram-negative cells and cell walls. Salton (1952, 1953) found that the walls of Gram-negative organisms contain considerably more lipid than the walls of Gram-positive organisms, while Mitchell & Moyle (1954) found that Gram-positive organisms possess only half the amount of lipid phosphorus found in Gram-negative organisms.

From the results described in this paper it is clear that the affinity of the small-particle fraction of *Bacillus megaterium* for DANSP is much greater than that of the cell walls. The redistribution observed on the addition of small particles to DANSP-treated cell walls demonstrates that studies of the absorption of polymyxin by isolated cell-wall preparations (Few & Schulman, 1953) give no indication of the distribution of polymyxin in the intact cell. Such studies have, however, given some indication of the nature of the resistance to polymyxin shown by many Gram-positive organisms (Few & Schulman, 1953) and by one strain of *Pseudomonas aeruginosa* (Newton, 1954*b*). Cell walls of resistant organisms absorb only about one-fifth the amount of polymyxin absorbed by the walls of sensitive organisms, and it seems possible that in the case of resistant organisms there is little penetration of polymyxin through the cell wall to the underlying membrane or osmotic barrier.

I wish to thank Dr E. F. Gale, F.R.S., for his continued interest and encouragement. Thanks are also due to Dr G. Weber for many helpful discussions and for a gift of 1-dimethylaminonaphthalene-5-sulphonyl chloride, and to Mr J. Smiles and Mr M. R. Young of The National Institute for Medical Research, Mill Hill, London, for taking fluorescence photomicrographs.

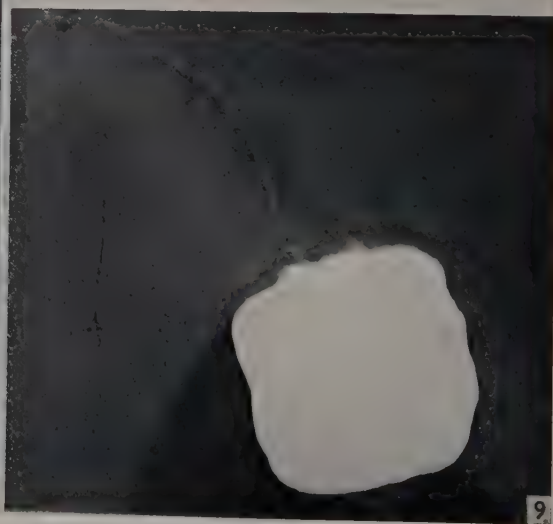
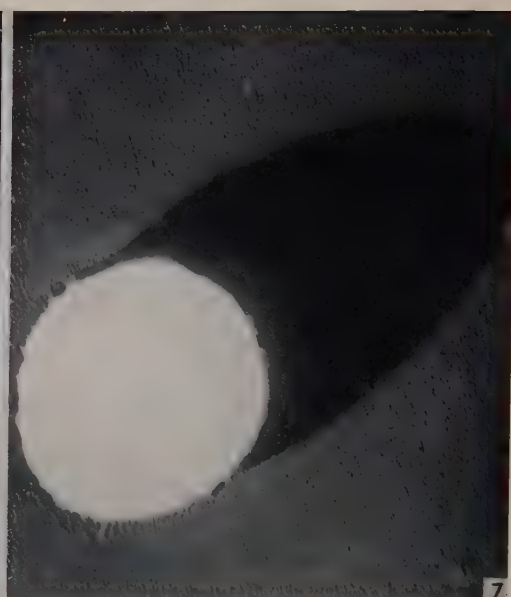
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B. A. NEWTON—A FLUORESCENT DERIVATIVE OF POLYMYXIN. PLATE 1

(Facing p. 234)



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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. Chromatographic separation of DANSP and 1-dimethylaminonaphthalene-5-sulphonic acid. Whatman no. 1 paper; solvent, molar phosphate buffer pH 7.0; fluorescence excited by mercury arc with Wood's filter. (1) DANSP after filtering through Dowex 2; (2) DANSP before filtering through Dowex 2; (3) 1-dimethylaminonaphthalene-5-sulphonic acid.
- Fig. 2. Fluorescence photomicrograph of *Pseudomonas aeruginosa* treated with 25 μ g. DANSP/mg. dry wt. cells. $\times 1050$.
- Fig. 3. Fluorescence photomicrograph of *Bacillus megaterium* treated with 25 μ g. DANSP/mg. dry wt. cells. $\times 700$.
- Fig. 4. Protection of cells by cations. (1) untreated cells; (2) cells treated with 25 μ g. DANSP/ml. for 5 min.; (3) cells suspended in 0.05M-calcium chloride before treatment with DANSP (25 μ g./ml.) for 5 min. Fluorescence excited by mercury arc with Wood's filter.
- Fig. 5. Fluorescence photomicrograph of DANSP treated (25 μ g./mg. dry wt. cells) *Bacillus megaterium* after depolymerization of the cell wall by treatment with lysozyme. $\times 700$.

PLATE 2

- Fig. 6. Washed cells of *Bacillus megaterium*. $\times 18,000$.
Fig. 7. Protoplast prepared from *Bacillus megaterium* by controlled lysozyme treatment; preparation fixed with mercuric chloride and formalin. $\times 26,950$.
Fig. 8. Protoplast membrane (*Bacillus megaterium*) resulting from lysis of protoplasts by suspension in distilled water; preparation fixed in mercuric chloride and formalin. $\times 26,950$.
Fig. 9. Sub-cellular unit obtained by lysozyme treatment of *Bacillus megaterium* which had been pretreated with a bactericidal concentration of polymyxin; preparation not fixed. No lysis resulted on repeated washing with distilled water. $\times 26,950$.

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The Effect of Partial Steam Sterilization on the Protozoan Fauna of a Greenhouse Soil

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SUMMARY: Comparison of the cultures from treated and untreated greenhouse soil showed that partial steam sterilization decreased the number of protozoan species, delayed the development of the fauna, but favoured the growth of the surviving ciliate species. This was particularly true of the treated topsoil.

In 1909 Russell & Hutchinson put forward the view that 'sick' soils owed their infertility to low bacterial activity, that bacterial activity was inhibited by some factor which was removed by partial sterilization, and that this inhibitory factor was the protozoan fauna. It is now realized, however, that partial sterilization, particularly by steam injection, has widespread effects on the soil equilibrium and that no one factor is sufficient to explain its beneficial effects (Anon., 1948). It is recognized that the total effect of steaming soil is more extensive than its biological effect alone and that physical and chemical characteristics of the soil are also changed. These include water-holding capacity, capillary properties, destruction of the colloidal film surrounding soil particles, increased concentration of the soil solution, and increased solubility of soil constituents (e.g. Ca, Mg, Mn, phosphate). The biological effects are an initial inhibition of bacterial activity, and seed germination followed by greatly stimulated bacterial and plant growth.

Singh & Crump (1953) examined the effect of partial sterilization on the numbers of amoebae in a field soil. In the present paper, the results of which agree generally with their findings, I am concerned with the effect of partial steam sterilization on the number of species in the samples and on ciliate growth.

METHODS

The samples examined were from a greenhouse soil at the Cawthron Institute which had been used for growing tomatoes. The soil was sterilized by steam injection to a depth of 18 in. by pipes inserted from above. Samples were taken 100 days later from the topsoil (0-4 in.) and subsoil (20-24 in.) of both treated and untreated soil. These samples were cultured 24 days later, in the following way and the development of the protozoan fauna followed.

Sterile 10 cm. Petri dishes were used with a plain agar base. A sample of soil (10 g.) was added to the side of a dish and then 20 ml. sterile water; incubation was at room temperature (c. 18°). For examination a plate was

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placed on the microscope stage and examined with the aid of low power and water-immersion lenses. Species were identified morphologically and the growth of the protozoan fauna followed in this way.

RESULTS

The cultures were set up on 11 October. After 1 day there was little protozoan activity; *Saprophilus* spp. appeared in the untreated topsoil culture and *Vorticella striata* in the untreated subsoil culture. After 2 days, flagellates were omnipresent, but whereas the treated subsoil showed no ciliates and the treated topsoil only a few *Colpoda inflata*, the untreated samples showed a number of species. The subsoil had *C. inflata*, *C. steinii*, *Saprophilus* spp., and a rotifer. The topsoil had these three ciliate species, together with *Oxytricha pellionella*, *Chilodonella* sp., and nematodes.

After 3 days, the treated subsoil had *Colpoda inflata*, and the treated topsoil added *Oxytricha pellionella* to its fauna as did the untreated subsoil. At this time the untreated topsoil was readily distinguishable by the greater variety of species present and still more by the greater number of individuals, especially *Colpoda inflata* and *C. steinii*, which far exceeded the numbers present in the other cultures.

After 11 days, *Chilodonella*, *Oxytricha*, *Vorticella*, *Euplotes* and *Trichopelma* spp. were noted in the untreated topsoil, the two *Colpoda* spp. having disappeared. In the untreated subsoil *Oxytricha*, *Vorticella*, *Enchelys*, *Mayorella* and *Sphenoderia* spp. were present. In the treated subsoil *Colpoda inflata*, *Oxytricha* and *Vorticella* spp. were present, while in the treated topsoil there were only two ciliate species, *Colpoda inflata* and *Oxytricha* sp. These were present, however, in very great numbers. With a lens combination L.P. $\times 10$, up to 100 individuals could be observed in one field anywhere on the plate.

Except for the occurrence of *Späthidium* sp. and *Lionotus* sp. in the treated subsoil, the subsequent history of the cultures was of no particular interest.

A full list of the fauna observed in the four cultures is given in Table 1.

DISCUSSION

These observations demonstrate several important facts. First, as shown in Table 1, the number of species in the treated topsoil was greatly decreased by the steaming; there were only six species compared with twenty-five in the untreated topsoil. The treated subsoil, however, was little affected; twenty-one species were found as compared with twenty-two in the untreated subsoil. Secondly, the protozoan fauna of the steamed soils appeared some days after that of the untreated soils; there was a time lag in multiplication. Thirdly, protozoan activity in the steamed soils was far greater than in the untreated soils after the fauna had developed. This was particularly true of the steamed topsoil. The two principal ciliate species which occurred in this culture were *Colpoda inflata* and *Oxytricha pellionella*. After 11 days of cultivation these ciliates were present in very great numbers which have never been paralleled by any other soil culture in my experience.

From these results it is obvious that while Russell & Hutchinson were correct in surmising that the protozoan fauna would be drastically decreased by partial sterilization, they were in error when they attributed the heightened activity of the bacteria to the absence of protozoa. For not only are protozoa present in the treated soil but they are present in very great numbers and

Table 1. *The protozoan fauna of steam treated and untreated greenhouse soil*

	Depth of sample (in.)			
	0-4	20-24	0-4	20-24
	Untreated		Treated	
Mastigophora				
<i>Bodo</i> sp.	×	×	.	×
<i>Monas</i> sp.	×	×	.	.
<i>Euglena</i> sp.	.	×	.	×
<i>Cercomonas</i> sp.	×	×	.	×
<i>Astasia</i> sp.	×	×	×	×
<i>Oikomonas</i> sp.	×	×	.	.
<i>Mastigameba</i> sp.	×	×	.	.
Rhizopoda				
<i>Mayorella vespertilio</i>	×	×	×	×
<i>Trichamoeba</i> sp.	×	.	×	×
<i>Cryptodifflugia oviformis</i>	×	×	.	×
<i>Trinema lineare</i>	×	.	.	×
<i>Sphenoderia dentata</i>	×	×	.	×
<i>Euglypha rotunda</i>	×	.	.	.
<i>Nuclearia simplex</i>	.	.	.	×
Ciliata				
<i>Spathidium</i> sp.	×	×	.	.
<i>Enchelys</i> sp.	×	×	?	.
<i>Chilodonella</i> sp.	×	×	.	.
<i>Lionotus</i> sp.	.	.	.	×
<i>Trichopelma sphagnetorum</i>	×	.	.	×
<i>Colpoda steinii</i>	×	×	.	.
<i>C. inflata</i>	×	×	×	×
<i>Colpidium</i> sp.	.	×	×	×
<i>Pseudoglaucoma</i> sp.	.	×	.	×
<i>Saprophilus</i> sp.	×	?	.	?
<i>Cinetochilum margaritaceum</i>	.	.	.	×
<i>Cyclidium glaucoma</i>	×	?	.	×
<i>Cohnilembus fusiformis</i>	×	×	.	×
<i>Oxytricha pellionella</i>	×	×	×	×
<i>Euplotes</i> sp.	×	×	.	.
<i>Vorticella striata</i>	×	×	.	×

their activity is stimulated by partial sterilization just as much as the activity of the bacteria. Both show an initial inhibition followed by greatly enhanced activity of that part of the flora and fauna which is not killed by the steaming.

Kidder & Stuart (1939*a, b*) found that the growth of *Colpoda steinii* was depressed in a mixed bacterial culture and that ultimately the ciliates died out. They also found that cyst formation was inhibited and that the ciliates which normally divide within a cyst membrane failed to do so. Singh (1941) confirmed the depressing effect which old culture filtrate had on the growth of

Colpoda steinii even when adequate food was available. He showed that the toxic effect of the filtrate was removed by heating for 1 hr. at 70–80°. Brown & Taylor (1938) found that excystment of *Colpoda* could be interrupted by returning cysts from their excystment medium to the medium in which they encysted. It appears that there is a thermolabile toxic factor in old culture fluid as a result of bacterial activity. The effect of steam sterilization is to remove this factor which will have the double effect of removing an excystment inhibitor and an inhibitor of ciliate growth. It will account therefore for the greatly stimulated growth of *Colpoda* spp. in the present cultures.

I am indebted to Dr E. B. Kidson of the Cawthron Institute for the opportunity to examine these soil samples.

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ISAACS, A. & DONALD, H. B. (1955). *J. gen. Microbiol.* **12**, 241-247

Particle Counts of Haemagglutinating Viruses

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SUMMARY: The electron microscope was used to make particle counts of the viruses of Newcastle disease of fowls, fowl plague, mumps and influenza C. Two counting techniques previously described were also used, and correlations with simultaneous measurements of haemagglutination and infectivity were made.

In a previous study (Donald & Isaacs, 1954*a*) counts were made in the electron microscope of influenza virus particles, and related to measurements of haemagglutination and infectivity of the preparations carried out under optimal conditions. It was found that at the agglutination end-point there was about one virus particle/red cell. It was also found that about ten virus particles of the standard influenza strains corresponded to the minimal infective dose. The particle count methods developed for that study are extended here to the related haemagglutinating viruses of Newcastle disease, fowl plague, mumps and influenza C. It was not always possible to use both counting techniques for each of these viruses; however, the modifications developed for each particular case and the results obtained are presented in this paper.

MATERIALS AND METHODS

Virus strains. Fowl plague—'Dutch' strain; Newcastle disease—(N.D.V.) 'Herts.' strain; mumps—Enders EMA 41 strain; influenza C—Taylor's 1233 strain (1949).

Both particle-counting techniques and the methods of biological assay were described in an earlier paper (Donald & Isaacs, 1954*a*). Certain modifications were introduced for each of the above viruses.

Preparation of virus material. With influenza C 10-day eggs were inoculated amniotically since this virus does not grow well in the allantoic cavity, and the allantoic fluid was harvested after 2 days. Dilutions of 10^{-4} of the seed virus were used for inoculation.

Newcastle disease and fowl plague viruses were passaged in the allantoic cavity of 10-day eggs and harvested after 18 hr. of incubation at 35°. A dilution of 10^{-4} of the seed virus was used for Newcastle disease virus, 10^{-3} for fowl plague.

Mumps virus was passaged in the allantois of 8-day eggs and harvested after 5 days of incubation at 35°. A dilution of 10^{-4} of the seed virus was used for inoculation.

Modifications to haemagglutination titration. Titrations with influenza C were carried out in the cold room (+2°) with pre-chilled reagents, as this virus elutes spontaneously from red cells at room temperature. Titrations with

Newcastle disease virus were read immediately on settling as this virus elutes quite rapidly at room temperature.

Modifications to infectivity titration. For influenza C titration in the amniotic cavity was used and eggs were incubated for 3 days at 35° after inoculation. Mumps virus was titrated in the allantoic cavity with incubation for 5 days after inoculation. Amniotic titration of mumps virus did not improve the sensitivity of the infectivity titration.

Modifications to red cell counting technique

Fowl plague virus. Absorptions were most successful with cells not treated with periodate and an absorption time of 15 min. at 0°.

Newcastle disease virus. Maximal absorptions were obtained after 1 hr. at 0° using about 2 ml. of a 10 % suspension of red cells/ml. infected allantoic fluid. This was approximately twice the concentration required for influenza virus strains of comparable haemagglutinin titre. Periodate treatment of red cell ghosts did not improve absorption.

Influenza C virus. The normal absorption techniques even with extreme precautions in regard to chilled reagents and centrifugation temperature did not cause significant absorption of viral haemagglutinins. A 'cascade' process similar to that advocated by Minuse, Quilligan & Francis (1954) was successful, however. The infected allantoic fluid was absorbed with red cells, and the supernatants absorbed with fresh red cells a number of times until the virus was satisfactorily removed. In a typical experiment using three lots of red cells and 5 min. absorption times at 0° it was possible to decrease the haemagglutination titre of a particular preparation from 1280 to less than 10, although the same amount of cells added in a single absorption had no detectable effect on the haemagglutinin titre. It was not possible to obtain accurate particle counts with this type of absorption technique so the method was used solely as a check for the specificity of the spraying technique. The supernatant at the end of the 'cascade' process was dialysed against distilled water, mixed with polystyrene latex particles and sprayed in the usual way.

Mumps virus. A similar 'cascade' process was found to be satisfactory. Six separate absorptions of 5 min. each at 0° were necessary before 80 % of the virus was absorbed. Again, this technique was used solely as a check that particles occurring in original sprayed droplets were the elementary bodies of the virus.

Modifications to spraying technique. Preparations of mumps and Newcastle disease virus were fixed with osmic acid before spraying. Fixation with 0.1 % osmic acid was found to have a deleterious effect on the elementary bodies of fowl plague virus. Unfixed preparations were therefore sprayed in an ultra-violet cabinet. For all spray counts the correlation coefficients between virus and latex particles were ≥ 0.6 with $P < 0.01$.

The four viruses, fowl plague, Newcastle disease, mumps and influenza C will be treated separately as there were differences in applicability of the two counting techniques, and also interesting morphological differences in the particles.

RESULTS

Fowl plague virus

Pl. 1, fig. 1 shows the results obtained with electron microscopy and the red cell absorption technique. The elementary bodies of the virus are mainly spheres of approximately 100 m μ . diameter associated with a few short filaments. The virus is very similar in appearance to standard influenza strains. Pl. 1, fig. 2, shows the results obtained with the spray technique. The virus particles seen in the droplet patterns were readily removed by absorption with red cell ghosts and did not appear in the patterns sprayed after absorption.

Counts were made by the two techniques of the numbers of particles/ml. and these counts were related to the infectivity and haemagglutination titres. Ratios for the number of particles/agglutinating dose (particles/AD), number of minimal infective doses/agglutinating dose (ID 50/AD), and number of particles/ID 50 were calculated and are shown as logarithms (base 10) in Table 1. There was no spray count for Expt. 1 as the dialysed material had

Table 1. Ratios (\log_{10}) of particles/agglutinating dose, egg infective dose/agglutinating dose, and particles/egg infective dose for fowl plague virus

The table shows results of three separate experiments.

	Ratio (\log_{10})		
	—	6.9	6.9
Particles/AD—spray technique	—	6.9	6.9
Particles/AD—r.b.c. technique	7.1	6.9	7.1
ID 50/AD	6.2	5.0	6.1
Particles/ID 50	0.9	1.9	0.9

been treated with osmic acid which made subsequent identification of particles very difficult. The type of result obtained is similar to that found previously with influenza virus strains, although the ratio of particles/agglutinating dose is slightly lower than that of standard influenza strains. The material used in the second experiment, although harvested at 18 hr., had an unusually low ratio of ID 50/AD. Otherwise the results as regards the ratio of particles/agglutinating dose are consistent and give an average figure of $10^{7.0}$. It seems likely that under optimal conditions there are close to 10 particles in the infective dose (ID 50).

Newcastle disease virus

Pl. 1, fig. 3, shows the results obtained with electron microscopy and the red blood cell absorption technique. We found a wide variation in particle size (approximately 150–250 m μ . in diameter) in agreement with previous workers (Dawson & Elford, 1949). There is some indication of internal structure after fixation with osmic acid. Pl. 1, fig. 4, shows the results obtained with the spray technique. The same wide variation in size distribution is noticed, and internal structures are quite clear. All particles identified and counted as

virus were removed by red cell absorption. The counts and ratios obtained by the two techniques are shown in Table 2. Some difficulty was experienced with the biological tests with this virus. Infectivity titrations, carried out 2 or 3 times on each material, tended to give 'takes' over a wide range of dilutions near the end-point, suggesting considerable egg variation in the

Table 2. *Ratios (\log_{10}) of particles/agglutinating dose, egg infective dose/agglutinating dose, and particles/egg infective dose for Newcastle disease virus*

	Three experiments.		
	Ratio (\log_{10})		
Particles/AD-spray technique	7.0	7.1	7.0
Particles/AD-R.B.C. technique	7.0	7.1	7.2
ID 50/AD	6.1	6.3	6.6
Particles/ID 50	0.9	0.8	0.7

response to infection by this virus. Also with some preparations of Newcastle disease virus there was far more variation in the results of haemagglutination titrations by the pattern method than with influenza strains; at present there is no explanation for this finding. When this difficulty was encountered it was assumed that the haemagglutination titre was too variable to use as a standard of reference. The ratios were therefore calculated where possible without including the haemagglutination titres.

The ratio of particles/AD is about $10^{7.1}$, again not greatly different from that of the influenza and fowl plague viruses. The ratio of ID 50/AD appears to be higher than in the case of influenza viruses particularly in Expt. 3. The ratio of particles/ID 50 is rather lower than for influenza virus strains, being in the range of 0.7–0.9 log units, i.e. 5–7 particles. This figure is in good agreement with the calculations of Bang (1948).

Mumps virus

The particles of mumps virus could be readily counted by the spray technique. A droplet pattern containing mumps virus and polystyrene latex particles is shown in Pl. 2, fig. 5. A wide variation in diameter (approximately 100–300 $m\mu$.) may be noted (cf. Dawson & Elford, 1949; Ray & Swain, 1954), and there are marked indications of internal structure which appear after osmic acid fixation. Pl. 2, fig. 6, shows a sprayed droplet after 'cascade' absorption has been carried out. Only latex particles are now present.

The particle counts and ratios obtained for three experiments are shown in Table 3. The ratio of particles/agglutinating dose for mumps virus was much higher than with any of the strains hitherto described. This fact may be related to the difficulty of absorbing mumps virus with red cells. High ratios of particles/agglutinating dose were also found with influenza C virus which is also difficult to absorb with red cells. This point is discussed further under influenza C virus.

Preparations 1 and 2 showed a lower ratio of ID 50/AD than was found with most preparations of influenza virus and this finding is characteristic of mumps

virus. The third preparation gave an unusually high ratio of ID 50/AD, although the techniques followed were not varied in the three experiments. Usually, however, mumps virus showed a low efficiency of initiating infection, some hundreds of particles/ID 50 being necessary whether titrated in the allantoic or amniotic cavity of the chick embryo.

Table 3. Ratios (\log_{10}) of particles/agglutinating dose, egg infective dose/agglutinating dose, and particles/egg infective dose for mumps virus

	Three experiments.		
	Ratio (\log_{10})		
Particles/AD-spray technique	7.4	7.4	7.5
ID 50/AD	4.5	4.9	6.3
Particles/ID 50	2.9	2.5	1.2

Influenza C virus

The spray technique was found most suitable for influenza C virus. Pl. 2, fig. 7, shows a sprayed droplet pattern containing particles of this virus. These are spheres of approximately 100 m μ . diameter, but are much more flattened (they cast very short shadows in the electron microscope image) and lacking in contrast than the strains of influenza virus previously studied. Clumps of virus particles such as those shown at higher magnifications in Pl. 2, fig. 8, occurred frequently, particularly in the second experiment. 'Cascade' absorptions removed both single particles and clumps from infected fluids. Only a few filaments were seen adsorbed to red cell ghosts. Counts were made of the number of individual particles, and in addition an attempt was made to count the number of particles in the clumps wherever this was possible. Table 4 gives the results obtained. The particle counts are

Table 4. Ratios (\log_{10}) of particles/agglutinating dose, egg infective dose/agglutinating dose, and particles/egg infective dose for influenza C virus

	Ratio (\log_{10})		
	7.4-7.4	7.5-7.7	7.6
Particles/AD-spray technique	5.6	5.7	6.2
ID 50/AD	1.8-1.9	1.8-2.0	1.4
Particles/ID 50			

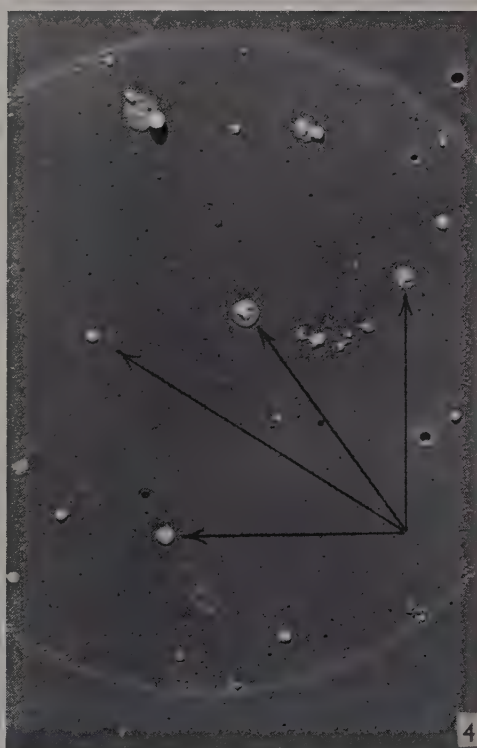
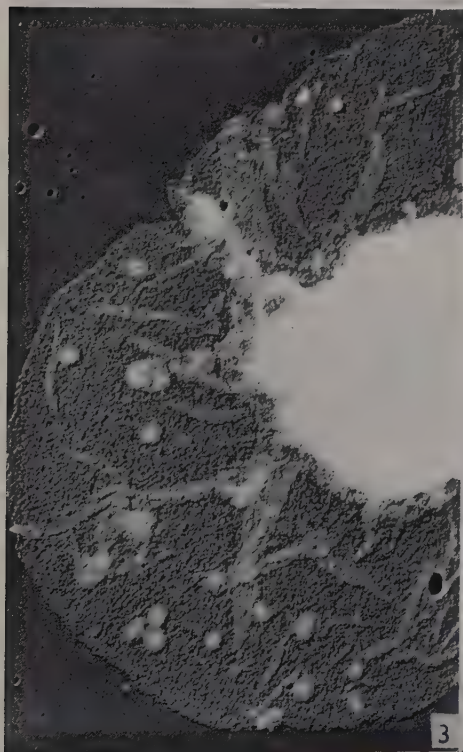
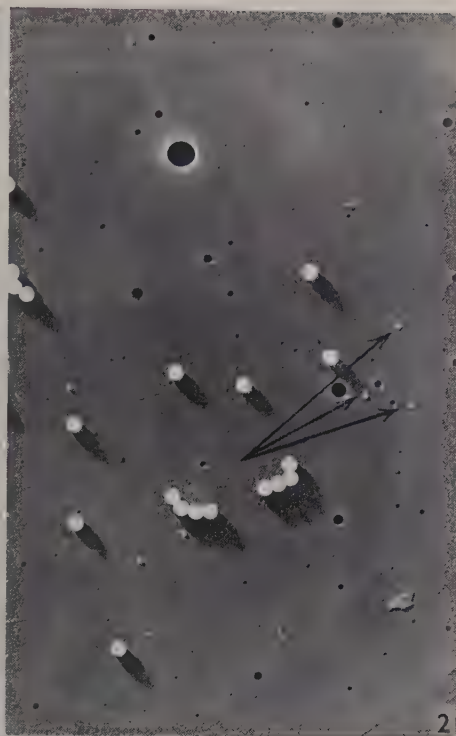
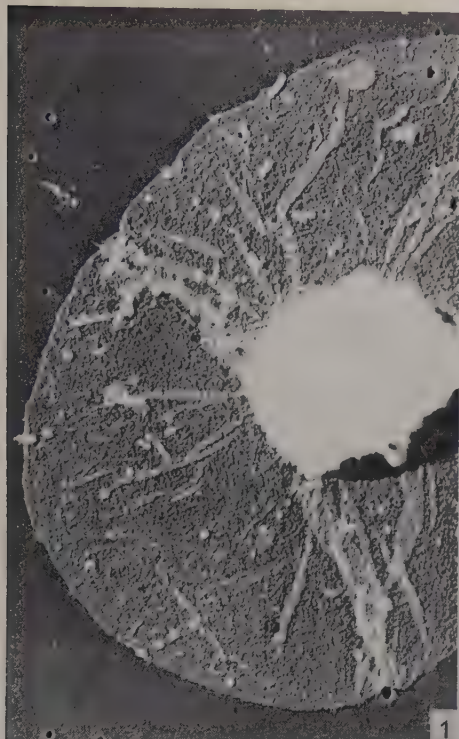
indicated in a range: in the higher figure given the particles within clumps are estimated individually, in the lower figure clumps have been counted as single 'particles'. As in the case of mumps virus the ratio of particles/agglutinating dose is higher than that of standard influenza strains. It seems likely that this high figure is a reflexion of the poor adsorption of this virus to fowl cells, as demonstrated by the need to use the 'cascade' technique to obtain efficient absorption. Thus one could visualize that at the haemagglutination end-point most of the virus particles present are not adsorbed to the fowl cells. There is no direct experimental evidence for this but the coincidence of high particle/AD ratios and poor adsorption to fowl cells found with both mumps and influenza C viruses is in favour of this explanation.

The correlation coefficient between latex and virus particles in Expt. 2 was below the level of statistical significance since the correlation found might have occurred by chance in approximately one out of ten experiments. This is the least significant correlation coefficient found in all the present experiments and may be due to the complicating factor of virus clumps, as about one-third of the particles counted in this experiment occurred in clumps of 3 to 50 particles. In Expts 1 and 3 there were fewer clumps and the correlation coefficients were statistically significant.

DISCUSSION

In this and two preceding studies (Donald & Isaacs, 1954*a, b*) particle counts were made of a number of strains of haemagglutinating animal viruses, and the figures related to the agglutination titres in a pattern test with $10^{7.1}$ to $10^{7.2}$ red cells. The figures found have been surprisingly constant and in most cases $10^{7.1}$ to $10^{7.2}$ virus particles/agglutinating dose has been the result, i.e. about one virus particle/red cell at the agglutination end-point (cf. Werner & Schlesinger, 1954). The exceptions found have been influenza virus filaments which gave a ratio of about $10^{6.6}$ and mumps and influenza C viruses which have given ratios of $10^{7.4}$ to $10^{7.7}$. The low ratio for influenza virus filaments means a much more efficient agglutination process than that found for spheres, and is presumably related to the greater average mass of viral haemagglutinin/particle in filamentous strains. Thus experiments with ultrasonic vibrations showed that virus filaments contained agglutinin along their entire length (Donald & Isaacs, 1954*b*), a finding which makes it easy to understand their more efficient agglutinating behaviour. Mumps and influenza C viruses, on the other hand, have shown a less efficient agglutinating behaviour than standard influenza strains, and it is probably not a coincidence that these are the only two strains we have studied which were not readily absorbed by fowl red cells, but required the 'cascade' absorption technique. Presumably, at the agglutination end-point with these two strains, only a small fraction of the virus is adsorbed to the red cells.

The ratio of infective doses/agglutinating dose (ID 50/AD) with these viruses has shown far more variation in our hands. The commonest figure found has been about $10^{6.1}$ ID 50/AD, but we have recently studied one strain of influenza virus with a ratio of nearly 10^7 , i.e. almost every particle was infective, whereas other strains tested under identical conditions have repeatedly shown figures of about $10^{5.0}$. With some strains of virus we have noticed big variations in this ratio in different experiments (e.g. mumps virus, Table 3). In searching for an explanation for these variations it seems important that we have used allantoic fluids from individual eggs in order to obtain the clearest fluids for electron microscopy; on the other hand, egg-infectivity measurements have frequently been carried out 2 or 3 times, generally with fairly reproducible results. This suggests that variability in the ID 50/AD ratio in the same strain may be due to variations in the quality of virus produced in individual eggs, and the behaviour of individual eggs



A. ISAACS & H. B. DONALD—PARTICLE COUNTS OF HAEMAGGLUTININATING VIRUSES. PLATE 1
(Facing p. 246)



A. ISAACS & H. B. DONALD—PARTICLE COUNTS OF HAEMAGGLUTININATING VIRUSES. PLATE 2

deserves further attention in this respect. The variation in ID 50/AD ratio with different strains, and with the same strain tested on different occasions in individual eggs, may be in some way related to the phenomenon of 'incomplete' virus, described by von Magnus (1946). Our results suggest that the differences in ratio are unrelated to the number of virus particles/agglutinating dose or to the efficiency of the adsorptive and agglutinating behaviour of different virus strains.

We wish to thank Mr O. Green and Mr E. Owen for their most valuable technical assistance.

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EXPLANATION OF PLATES

All preparations were gold-manganin shadowed.

PLATE 1

- Fig. 1. Particles of fowl plague virus absorbed on to a red cell ghost. $\times 10,000$.
- Fig. 2. Part of a microdrop containing polystyrene latex and fowl plague virus particles. The small flatter particles indicated by arrows are the elementary bodies of the virus. $\times 10,000$.
- Fig. 3. Particles of Newcastle disease virus absorbed on to a red cell ghost. $\times 10,000$.
- Fig. 4. Part of a microdrop containing polystyrene latex and Newcastle disease virus particles. The particles indicated by arrows are the elementary bodies of the virus. $\times 10,000$.

PLATE 2

- Fig. 5. Part of a microdrop containing polystyrene latex and mumps virus particles. The particles indicated by arrows are the elementary bodies of the virus. $\times 10,000$.
- Fig. 6. Part of a microdrop showing allantoic fluid infected with mumps virus after 'cascade' absorption with red cells. Polystyrene latex particles but no mumps virus particles present. $\times 10,000$.
- Fig. 7. Microdrop containing polystyrene latex, and particles of influenza C virus indicated by arrows. $\times 10,000$.
- Fig. 8. Same as fig. 7 but $\times 25,000$. In addition to flattened isolated particles, a clump is shown.

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The Formation of Extracellular Nitrogen Compounds by Fungi

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SUMMARY: The production of nitrogen compounds in the culture medium by *Scopulariopsis brevicaulis* and other fungi has been studied. The formation of extracellular nitrogen compounds accompanied the primary assimilation of ammonia, nitrate and organic nitrogen in all experimental conditions so far investigated among the various factors studied. The amount of these extracellular nitrogen compounds was affected markedly only by the supply of certain trace elements. It was lowest when the concentration of trace elements was high, and increased as the concentration of trace elements approached that limiting growth. Most of the extracellular nitrogen appeared to be peptide in nature, yielding some 14 amino acids on hydrolysis. The fungus was unable to assimilate the extracellular nitrogen compounds formed but assimilated the constituent amino acids when these were liberated by acid hydrolysis.

When fungi grow on inorganic nitrogen sources organic nitrogen compounds appear in the culture medium at the same time (Morton, 1951). This phenomenon seems to be frequent among micro-organisms; it has been observed in bacteria (Fedorov, 1952; Hotchkiss, 1950; Proom & Woiwod, 1949), in blue-green algae (Fogg, 1952; Henriksson, 1951; Watanabe, 1951) and in fungi by many authors (Dietzel, Behrenbruch & Eucken, 1951; Hockenhull, 1950; Iwanoff & Krupkina, 1929; Nielson & Hartelius, 1937; Reindel & Hoppe, 1952; Steinberg, 1939). The formation of extracellular nitrogen compounds by fungi always occurs during the period of active assimilation of nitrogen, as Nielson & Hartelius (1937) noted in experiments with yeast. This link with primary nitrogen metabolism is the most significant feature of our observations and prompted further investigation. These secondarily formed organic nitrogen compounds which appear in the medium during growth will for the sake of brevity be termed simply 'extracellular nitrogen'.

EXPERIMENTAL

The fungi used were grown in surface culture in small flasks containing 20 ml. of culture medium of the following basal composition: 50 g. glucose; 1.0 g. KH_2PO_4 ; 0.5 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; trace elements (200 μg . Fe, 38 μg . Cu, 225 μg . Zn, 24 μg . Mn, and 40 μg . Mo); water to 1 l. Different nitrogen sources were added as required. The nitrogen source was always sterilized separately from the other components of the medium in order to avoid any interaction with the carbohydrate present. The pH value of the media was adjusted to 6.5 initially, and the cultures were incubated at 25°.

Replicate flasks were harvested at intervals after inoculation. The culture medium was gently washed out, made up to standard volume to allow for evaporation, and filtered to remove mycelium and spores. Estimations of ammonia and nitrate were carried out on Conway micro-diffusion units by methods previously detailed (Morton & MacMillan, 1954). Total nitrogen was determined by micro-Kjeldahl. Amino nitrogen was determined as ammonia liberated by the action of ninhydrin (MacFadyen, 1944). The ammonia was estimated by Nessler's reagent after vacuum distillation following the method of Archibald (1943).

For most of the experiments a strain of *Scopulariopsis brevicaulis* (Sacc.) Bainier (No. 55/OS/A1) was used. Other species used were *Aspergillus niger* v. Tieghem, *Penicillium chrysogenum* Thom, *Trichoderma viride* Pers. ex Fries, *Botrytis allii* Munn.

RESULTS

General features of nitrogen assimilation

The normal course of development in *Scopulariopsis brevicaulis* is illustrated graphically in Fig. 1 where the results from a number of separate experiments are assembled. In all experiments 6.6 mg. N (as ammonium sulphate)/flask

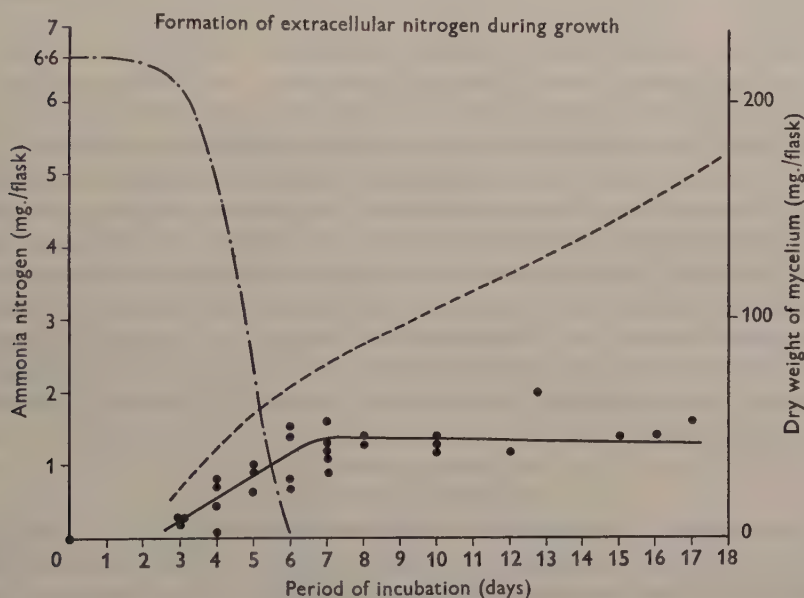


Fig. 1. Formation of extracellular nitrogenous material during growth (ammonia as N source). Dry weight ----; ammonia nitrogen, -.-.-; extracellular nitrogen, —.

was present initially. The main carbon source was glucose, but 1% (w/v) sodium malate, succinate or citrate was added to prevent adverse pH changes (see Morton & MacMillan, 1954). It will be seen that 'organic' nitrogen in the medium rose rapidly from about the 3rd to the 6th or 7th day, precisely during

the time when ammonia-nitrogen was most rapidly disappearing. After exhaustion of the ammonia the concentration of 'organic' nitrogen remained constant (within the limits of experimental error) up to 20 days and even longer.

The amount of glucose in the basal medium was more than sufficient to allow assimilation of all the nitrogen supplied. Indeed the dry weight of the fungus increased steadily at the expense of glucose for some time after all the ammonia had disappeared. The dry weight of mycelium roughly doubled itself between 7 and 20 days. There was no evidence of autolysis (recognized by the re-appearance of free ammonia in the medium) in this period. Microscopic examination showed that this secondary increase in dry weight was due to new growth and not simply to an accumulation of materials in the mycelium already formed. This growth was not the expression of conidia formation alone, although the conidia were mainly produced at this stage; there was, however, a considerable formation of new vegetative hyphae.

Since no measurable quantity of the extracellular organic nitrogen was used, it seems clear that there must be extensive re-mobilization of the internal nitrogen of the mycelium during this secondary growth period. Evidence of such re-mobilization was seen in the appearance of the mycelium. The cytoplasm in the older hyphae became highly vacuolate, whilst new hyphae grew out and were narrower in diameter and filled with dense scarcely vacuolate cytoplasm.

Thus, in normal cultural conditions, when glucose supply was not limiting, at least two distinct phases of nitrogen metabolism were observed. The first phase involved the assimilation of the initially added ammonia, and was accompanied by the formation of extracellular nitrogenous compounds. The second phase, judging by the mass of new hyphae formed, was an extensive re-mobilization of the internal nitrogen of the mycelium, accomplished without measurable quantitative change in the extracellular nitrogenous material already formed. There was no detectable mobilization of extracellular nitrogen, although glucose and other constituents of the culture medium were present in adequate amounts, as demonstrated by the fact that further additions of ammonia or nitrate were readily assimilated. The possibility of a balanced turn-over of extracellular nitrogen is not excluded, however, although it seems unlikely.

Quantitative aspects

The formation of extracellular nitrogenous material has been observed in a number of fungi and appears to occur generally. It is apparent from Tables 1 and 2 that the proportion of initially available nitrogen converted into extracellular nitrogen varied with the fungal species and the nature of the nitrogen source. The amount of extracellular nitrogen may represent a surprisingly high proportion of the nitrogen supplied initially. In *Scopulariopsis brevicaulis* 20–25 % of the nitrogen was finally found in this extracellular form whether the nitrogen source was ammonia or nitrate. Experiments were therefore carried out to see whether the concentration of extracellular nitrogen could be altered by changes in nutritional or other environmental conditions.

Table 1. Amount of nitrogen assimilated which appears in the medium after 7 days growth

Fungus	Nitrogen source (in basal medium)	Extracellular N (as % initially added N)
<i>Aspergillus niger</i>	Ammonia	7.5
	Nitrate	3.5
<i>Penicillium chrysogenum</i>	Ammonia	20.0
	Nitrate	7.5
<i>Trichoderma viride</i>	Ammonia	27.0
	Nitrate	36.0
<i>Botrytis alii</i>	Ammonia	23.5

Table 2. Formation of extracellular nitrogen in relation to initially added nitrogen which disappeared

Fungus	Nitrogen source	Amount of N supplied (mg./flask)		
		6.6	13.2	6.6 + 6.6
		Extracellular N as % N assimilated		
<i>Scopulariopsis brevicaulis</i>	Ammonia	25.35 ± 5.58	25.80 ± 2.86	.
	Nitrate	20.03 ± 4.47	16.62 ± 2.08	16.50 ± 1.98
<i>Penicillium griseofulvum</i>	Nitrate	13.00 ± 3.55	12.50 ± 0.70	12.34 ± 2.73

Change in concentration of nitrogen supplied. The effect of different nitrogen concentrations in the culture medium was tested. The amount of nitrogen in the culture medium was doubled, the concentration of glucose and salts being raised sufficiently to ensure complete assimilation of the extra nitrogen. In some experiments twice the usual concentration of nitrogen (as ammonia or nitrate) was added initially. In other experiments the fungus was grown on basal medium with the usual amount of ammonia or nitrate until this was just exhausted (6–7 days); then a second quantity of nitrogen compound was added, and growth allowed to continue until all the added nitrogen had again disappeared. The results of several experiments are summarized in Table 2. The amount of extracellular nitrogen formed is seen to be almost directly related to the amount of nitrogen assimilated, irrespective of whether the nitrogen was presented in one or in two doses.

In some other experiments the glucose concentration was decreased to 0.5% (w/v), leaving the nitrogen source at its usual concentration. With this smaller concentration of carbohydrate *Scopulariopsis brevicaulis* was able to assimilate only two-thirds of the added nitrogen (ammonia) in the medium, yet 15% of the nitrogen assimilated appeared as extracellular nitrogen. These results emphasize the close relation which exists between primary nitrogen uptake and the formation of extracellular nitrogen.

Nature of carbon source. Since glucose can form compounds with ammonia fairly readily it seemed possible that the formation of extracellular organic nitrogen might be related to the presence of glucose. However, when glucose

was replaced by 5% sodium acetate (known to be a good carbon source for *Scopulariopsis brevicaulis*), the nitrogen (ammonia) in the medium was completely assimilated and the usual proportion of it (18%) appeared as extracellular nitrogen.

Growth factors and inhibitors. *Scopulariopsis brevicaulis* was grown with and without a vitamin supplement containing biotin, thiamine, pyridoxin, nicotinic acid and inositol. The amount of extracellular nitrogen formed was exactly the same in both series.

The effect of a number of inhibitors and antibiotics was tested, including iodoacetate, coumarin, griseofulvin, aurantiogliocladin, whilst in other experiments growth was affected by diminishing the amount of sulphur or phosphorus in the medium to concentrations at which they became limiting. The results will not be reported in detail since none of the treatments altered significantly the proportion of extracellular nitrogen to nitrogen assimilated, although in several experiments the actual amount of nitrogen assimilated was considerably decreased.

Volume of culture medium. The proportion of extracellular nitrogen formed was not significantly altered by dissolving the basal amount of nitrogen source and other components of the medium in one half and in twice the usual volume of fluid. The fungus was grown in submerged (shaken) culture in order to test the effect of dilution with the least possible complication from variation in surface/volume ratio. The proportion of extracellular nitrogen to initially added nitrogen was practically the same in shaken and in surface culture.

Trace elements. The basal medium was normally supplemented by the addition (1 ml./l.) of a solution containing the trace elements iron, copper, zinc, manganese and molybdenum, at concentrations which experience indicated to be adequate for the growth of most fungi. In some experiments the concentration of trace elements was varied by adding multiples of the normal amounts. The effects on the formation of extracellular nitrogen (in a nitrate medium) are shown in Table 3.

Table 3. *Formation of extracellular nitrogenous material by Scopulariopsis brevicaulis with nitrate as added N source and with different concentrations of trace element mixture*

Trace element addition	Extracellular nitrogen (as % initially added nitrogen which disappeared)		Dry matter (mg.) at 10 days (all NO ₃ taken up from medium)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
No addition	32.0	26.0	181	198
Normal dose*	16.0	16.5	192	192
Normal dose × 2	13.0	.	186	.
Normal dose × 10	7.0	9.0	192	192
Normal dose × 20	.	7.0	.	204
Normal dose × 50	.	7.0	.	.

* The normal dose of trace elements is equivalent to the addition of 200 µg. Fe, 38 µg. Cu, 225 µg. Zn, 24 µg. Mn, and 40 µg. Mo, per litre of medium.

The extracellular nitrogen was highest when no trace elements were added, when the fungus must rely on the amounts of various elements present as impurities in the constituents of the medium, coming from the glassware, or introduced with the inoculum. No special precautions to exclude trace elements were taken in these experiments. The amount of extracellular nitrogen was decreased by 40–50 % by addition of the usual supplement of trace elements. The addition of 10 times the usual supplement caused a further decrease (c. 50 %) in the amount of extracellular nitrogen, but further additions had no effect.

Even at the lowest concentration of trace elements (no addition) all the nitrate nitrogen was completely assimilated, and there was no decrease in final dry weight of mycelium. The only evidence that the supply of trace elements might have been limiting was the fact that at the lower concentrations (no addition and normal dose) the rate of assimilation of nitrate and the rate of dry-matter accumulation were distinctly slower during the first 5 days of growth. There were no differences in rate of nitrate assimilation and dry-matter accumulation between cultures receiving 2 to 50 times the normal dose of trace elements. Further experiments will be required to determine the extent to which the effects on the yield of extracellular nitrogen are related to the concentration of individual trace elements. It is clear, however, that more extracellular nitrogen was formed when the supply of trace elements was decreased. On the other hand, even with relatively high concentrations of added trace elements the formation of extracellular nitrogen was not suppressed altogether. The possibility that some further trace element, not intentionally supplied, may be involved clearly requires investigation.

Organic nitrogen sources. The question arose whether extracellular nitrogen was produced when nitrogen was supplied initially in an organic form instead of as ammonia or nitrate. *Scopulariopsis brevicaulis* was grown on the basal medium plus various single amino acids, namely glycine, L-glutamic acid, L-arginine, L-alanine, γ -aminobutyric acid; enzyme-hydrolysed casein was also tested. Growth was vigorous on all amino acids tested except arginine, and the added single amino acids added disappeared, as shown by paper chromatography. Even with arginine, where growth was much slower, all this amino acid eventually disappeared. In every case nitrogen equivalent to 14–24 % of that added initially was found in the medium, but this nitrogenous material was different in chemical nature from that supplied initially (see below) and evidently represented extracellular nitrogenous compounds formed during metabolism (growth). The use of amino acids as nitrogen source thus involved the formation of extracellular nitrogenous compounds just as when inorganic nitrogen sources (ammonia, nitrate) were used.

The nature of the extracellular nitrogenous material

The chemical nature of the extracellular nitrogenous material was investigated in culture fluid of *Scopulariopsis brevicaulis*, taken after 7 days growth when the concentration of nitrogen in the medium had become constant, and all the ammonia or nitrate added initially had disappeared. There

were no differences in the general character of extracellular nitrogenous material whether the nitrogen source was ammonia, nitrate or an amino acid.

A large volume of culture filtrate was evaporated under reduced pressure to about one-eighth its original volume. No material was precipitated during this evaporation, or after treatment with trichloroacetic acid, phosphotungstic acid or other protein precipitants. Between 92 and 95 % of the nitrogen was dialysable against running water in 48 hr. Not more than 5 % of the extracellular nitrogenous material can therefore be protein in nature (extracellular enzymes).

The extracellular nitrogenous material gave reactions with ninhydrin for amino nitrogen suggesting the presence of free amino acids. Quantitative estimation of amino nitrogen showed that not more than 10 % of the extracellular nitrogen could be present as the α -amino groups of free amino acids. The remaining nitrogen (80–85 % of the total) did not react with ninhydrin directly. After hydrolysis with N-HCl at 100° for several hours, however, considerable quantities of amino nitrogen (reacting with ninhydrin) were liberated, which would suggest that peptide material was originally present. On estimation this liberated amino nitrogen was found to account for 60–80 % of the nitrogen of this fraction. Some of the amino acids liberated on hydrolysis were basic and contained nitrogen not estimated by ninhydrin. After making reasonable allowance for this it seems probable that there was some nitrogen (perhaps 10–20 % of this fraction) not to be accounted for as peptide.

Investigation of the nature of the extracellular nitrogenous material was carried further by paper chromatography. The presence of free amino acids in the culture fluid was confirmed and the main ones present at the early harvests (5–7 days) were identified as alanine, glutamic, aspartic and γ -aminobutyric acids. Traces of glycine, serine, proline, valine and leucine were also present and tended to become more distinct in later harvests (14–20 days). Rough estimates of the amounts of free amino acids were made from the chromatograms and confirmed that less than 10 % of the extracellular nitrogen occurred as free amino acids.

Examination of the main (peptide) extracellular nitrogen fraction after hydrolysis shows the presence of 14 amino acids: alanine, glutamic acid, aspartic acid, γ -aminobutyric acid, glycine, serine, threonine, proline, arginine, histidine, lysine, valine, tyrosine, leucine, and (probably) β -alanine. The first three amino acids listed were present in slightly larger amounts relative to the others but there was no marked preponderance of any one amino acid. Little more can be said at present about this peptide fraction. When the unhydrolysed material was examined by paper chromatography certain slow-moving indistinct spots which reacted very faintly with ninhydrin appeared on the chromatogram. When these spots were cut out and hydrolysed they yielded a range of amino acids. Attempts to resolve the spots by running solvent for long periods were not conclusive, and it remains uncertain whether the peptide fraction represents mainly a single polypeptide or a group of closely related peptides which tended to move together on the chromatogram.

The amino acid composition of different samples of the peptide fraction, as

far as it is possible to judge from chromatograms of the hydrolysate, shows little variation either in the number of amino acids present or in their relative proportions. No significant changes in composition were obvious either with time (7–20 days) in a given experiment, or with variations in cultural conditions (nitrogen source, carbon source, etc.).

Nitrogen in the mycelium

Some interest attaches to the question of the relation between the internal nitrogen of the mycelium and the extracellular nitrogenous material. Mycelium was extracted with a solution of 70 % (v/v) ethanol in water. After concentration of the extract total-N and amino-N were determined, and the material examined by paper chromatography. Free amino nitrogen accounted for 44 % of the total nitrogen extracted from the mycelium. Qualitatively there was a rather close relation between the free amino acids of the mycelial extract and the free amino acids of the extracellular nitrogenous material. Thus at 5 days the main amino acids in the mycelial extract were alanine, glutamic acid, aspartic acid and γ -aminobutyric acid, and these were the most prominent also in the culture medium. At later stages (14 days) several other amino acids appeared in the mycelial extract, although at much lower concentrations than the main ones, and the same acids were then detected in the culture medium. Quantitatively, however, there was a great difference between amounts of various amino acids in the mycelium and in the metabolism fluid; the ratio of the concentration of free amino acids in the mycelium to concentration in the metabolism fluid was c. 150:1 at 5 days and 100:1 at 14 days. When the material extracted by 70 % (v/v) ethanol in water was hydrolysed there was an increase in free amino-N corresponding to 6–7 % of the total N. This presumably represented peptide nitrogen extracted in the given conditions.

Physiology of the formation of extracellular nitrogenous material

In the usual experimental conditions the extracellular nitrogenous material appeared not to be metabolized further when the carbohydrate supply and other conditions would seem to have been favourable. It was thus of interest to determine whether the fungus could utilize some or all of its own extracellular nitrogenous material in different conditions. Since *Scopulariopsis brevicaulis* grows readily with certain single amino acids as N source, its ability to use the free amino acids of the extracellular nitrogenous material was first examined. Culture fluid from a 15-day growth of *S. brevicaulis* was passed through a column of a sulphonated polystyrene resin which retained the free amino acids. These were then eluted from the resin and used as the nitrogen source (at the usual concentration of N) when added to the basal medium. *S. brevicaulis* grew well on this medium, obviously utilizing the nitrogen, and after 14 day growth only 24 % of the nitrogen added remained in the medium; this amount did not differ significantly from the amount left when pure L-amino acids were used.

It is clear that the free amino acids of the extracellular nitrogenous material (after concentration) could be used by the fungus. They only make up 10 % of the extracellular nitrogen, however, whilst their concentration in the metabolism fluid was less than 1/100 of the free amino-acid concentration in the mycelium (calculated from the fresh weight). Possibly this low concentration of amino acids in the metabolism fluid represented a leakage from the mycelium: in other words, the hyphal permeability was such that the concentration of amino acids in the medium could not be decreased below a certain value. The close qualitative relation found between the amino acids of the mycelial extract and of the metabolism fluid would be in agreement with this view.

To test assimilability of the peptide fraction, culture filtrate from 15-day *Scopulariopsis brevicaulis* was evaporated down to suitable volume at a low temperature, and was used as the nitrogen source (at the usual concentration of 6.6 mg. N/flask), in fresh culture medium; the free amino acids were not removed. One set of flasks was inoculated with *S. brevicaulis* and others, for comparison, with *Penicillium griseofulvum* and *Aspergillus niger*. The culture filtrate was not toxic, as was shown by the excellent growth of all three fungi in flasks containing similar medium + potassium nitrate.

All three species made some growth when the extracellular nitrogenous material of *Scopulariopsis brevicaulis* was used as nitrogen source. The proportion of the initially-added nitrogen which was left in the culture medium after 15–20 days' incubation was 65 % for *S. brevicaulis*, 58 % for *Penicillium griseofulvum*, and 52 % for *Aspergillus niger*. Some 10 % of the initially added nitrogen was present as free amino acids, and could be assimilated. Therefore, of the peptide material between 25 and 40 % of its nitrogen appeared to be assimilated in these conditions. These figures are only approximate since they neglect the formation of more extracellular nitrogenous material. It is not certain that some change in the extracellular peptide nitrogen, making it partly assimilable, did not take place during evaporation of old culture fluid and the preparation of the new medium containing it. Nevertheless, it seems probable that 50–60 % of the extracellular nitrogen was not further metabolized by *Scopulariopsis brevicaulis*.

Finally the ability of the fungi to utilize the products of acid hydrolysis of extracellular peptide was tested. Culture filtrate from 15-day incubation of *Scopulariopsis brevicaulis*, after concentration, was hydrolysed for 18 hr. with 2N-HCl at 60°. The dark hydrolysate was treated with acetic acid-treated charcoal (no nitrogen removed) and after neutralization was used as nitrogen source in fresh culture medium. Flasks were inoculated with the same three fungi as before. Control flasks with acid hydrolysate + nitrate showed that the hydrolysate was not toxic.

All three fungi grew better than on unhydrolysed extracellular nitrogenous material; the proportion of initially added nitrogen which remained in the medium after growth was 40 % for *Scopulariopsis brevicaulis*, and 34 % for *Penicillium griseofulvum* and *Aspergillus niger*. Allowing for a fresh formation of extracellular nitrogenous material and for the occurrence of some non-

peptide nitrogen, these figures suggest that the amino acids derived from the peptide material by acid hydrolysis were assimilated normally. Thus the non-assimilability of the peptide material cannot be ascribed to the presence in it of D-amino acids, but appears to depend on its general structure. This is analogous to the case of naturally occurring toxic peptides, where toxicity is bound up with total structure and not with the occurrence of occasional D-amino acids or specific chemical groups.

DISCUSSION

The experiments described emphasize the regular connexion that exists between the disappearance of the initial nitrogen compound added (e.g. ammonia, nitrate) and the formation of extracellular nitrogenous material by fungi, and show that this extracellular nitrogenous material is not simply a product of autolysis or senescence. The proportion of initially added nitrogen which reappeared in the culture medium remained unchanged over a wide range of experimental conditions but was significantly affected by the concentration of trace elements supplied. In none of our experiments was the concentration of trace elements low enough to limit the final dry weight of the fungus or to prevent the assimilation of all the nitrogen supplied. Nevertheless, when judged by the initial rate of nitrogen uptake and dry-matter increase it seems that the concentration of trace elements normally used was suboptimal. It is significant that extracellular nitrogen was formed in greatest amount when the supply of trace elements shows signs of becoming limiting, suggesting a diversion of nitrogen caused by nutritional deficiency or imbalance. Extracellular nitrogen continued to be formed, however, even in conditions when the concentration of trace elements seemed unlikely to be limiting.

In all the experimental conditions used the general chemical nature of the extracellular nitrogenous material formed remained the same. A small proportion consisted of free amino acids, but the bulk of the material was probably peptide in character. It gave rise to a number of amino acids on hydrolysis, and these accounted for 80–100 % of the nitrogen of this fraction.

The presence of free amino acids in the culture medium during growth was noted by Mackenzie & Cook (1951) for *Penicillium notatum* and by Rao & Venkataraman (1952) for *P. chrysogenum*. The latter authors found accumulation of amino acids at first, followed by their utilization during later stages of growth. They did not look for peptide nitrogen. Reindel & Hoppe (1952) showed that extracellular nitrogenous material from yeast contained material which yielded 11 amino acids on hydrolysis, as well as free amino acids and unidentified nitrogen compounds. The proportion of free amino acids found (50 %) was considerably higher than for *S. brevicaulis*. Reindel & Hoppe found that not more than 20 % of the extracellular nitrogen could be re-utilized by a fresh yeast inoculum. This is in agreement with our results with mould fungi.

It must be said that our experiments do not yet throw much light on the physiological significance of the extracellular nitrogenous material. The

possibility has been considered that these nitrogen compounds may represent intermediates in protein synthesis which can only be utilized at a particular stage of growth and which tend to accumulate in certain conditions of nutritional imbalance. This interpretation does not seem very probable, however, in view of the evidence that this extracellular nitrogen is scarcely utilized by a fresh inoculum of the fungus even in a favourable medium. The problem seems most likely to be resolved by a parallel study of the changes in intracellular and extracellular nitrogen fractions during the course of nitrogen assimilation, especially in relation to the effects of the trace elements.

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Observations on the Morphology of *Streptomyces griseus* in Submerged Culture

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SUMMARY: The effects are described of three different media, based on urea, glycine or meat casein digest as nitrogen sources, on the morphology of *Streptomyces griseus* when grown in submerged culture. The urea medium was characterized by an alternation of morphological generations similar to that described by Klieneberger-Nobel for surface cultures: 'primary mycelium' alternated with 'secondary mycelium', the two generations being separated by an 'initial cell' stage. 'Secondary mycelium' sporulated to give 'secondary mycelium spores'. The other two media did not support 'initial cell' formation, but led either to the development of intercalary 'chlamydospores' or lysis without sporulation. The media based on urea or glycine were used in convenient and satisfactory techniques for the production of ampoules containing large numbers of lyophilized spores which stored satisfactorily.

Klieneberger-Nobel (1947) described an alternation of morphological generations in the surface culture of several *Streptomyces* species. The spores on germination gave substrate or 'primary' mycelium, which underwent anastomoses resulting in the formation of 'initial cells'. Klieneberger-Nobel regarded these 'initial cells' as fusion cells which on germination gave aerial or 'secondary' mycelium. This 'secondary mycelium' eventually sporulated to yield spores which completed the life-cycle by developing into new cultures of 'primary mycelium' on transfer to fresh medium. Erikson (1949), however, criticized Klieneberger-Nobel's interpretation of these observations on the grounds that the 'initial cells' and the 'nests' in which they formed were artefacts produced by the technique employed (stained coverslip growths) and that, in fact, the aerial filaments developed by budding from any vegetative hypha. While working on the development of media for the submerged cultivation of *Streptomyces griseus* we observed (1953) in certain media morphological forms which resembled those described by Klieneberger-Nobel for surface culture. Since our conditions differed greatly from those of Klieneberger-Nobel's surface culture methods, it seemed improbable that the morphological forms observed could be dismissed as artefacts. More detailed observations were therefore made and are reported here.

METHODS

The three types of media used contained urea, glycine or meat extract casein digest as the chief source of nitrogen, and had the following compositions:

The urea medium contained the following ingredients (g./l.): glucose (AnalaR), 5; sucrose (brown sugar), 10; urea (B.P.), 2; KH_2PO_4 (AnalaR), 2. This medium was adjusted, when necessary, to pH 4.9-5.0 with 5% phos-

phoric acid and sterilized in 400 ml. amounts in 2 l. round flat-bottomed flasks by autoclaving at 15 lb./sq. in. for 20 min. The post-sterilization pH value was 6.8–7.1. The brown sugar was not utilized by *Streptomyces griseus* but was included to provide traces of minor elements and organic substances.

The glycine medium was modified from a medium described by Lumb (1949) so as to provide a lower pH value during growth, and contained the following ingredients (g./l.): glucose monohydrate (pure Manbré & Garton), 20; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (AnalaR), 10; sodium citrate (AnalaR), 1; NaCl (AnalaR), 2.5; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (AnalaR), 0.87; KH_2PO_4 (AnalaR), 0.5; glycine (technical grade), 5; trace elements solution 10 ml./l. (The trace elements solution, used at its natural pH of about 5.4–5.5, consisted of (g./l.): $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.75; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.81; H_2MoO_4 , 0.17; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.10; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.44). The pH of the complete medium was adjusted to 7.0–7.1 with KOH before autoclaving 400 ml. volumes in 2 l. round flat-bottomed flasks at 15 lb./sq.in. for 20 min. The post-sterilization pH was 6.5–7.0.

The meat extract + casein digest medium was prepared from (g./l.): Lab-Lemco (Oxoid), 3; glucose (AnalaR), 10; casein digest (Benger's), 10. The pH value was adjusted to 7.0–7.2 with NaOH before filtering the medium through paper pulp, and 400 ml. volumes of the medium were then autoclaved at 15 lb./sq.in. for 20 min. in 2 l. round flat-bottomed flasks.

Microscopical examination. Material was smeared on a slide and fixed by gentle heat. Preparations were stained in basic fuchsin (1 % aqueous solution of Gurr's stain for 5–10 sec.), washed thoroughly with water and then destained in tannic acid (10 % aqueous solution of B.P. acid for 2 min.) to give a rose pink colour. The slides were finally washed thoroughly in water, dried and mounted in Canada balsam.

Lyophilization. When sporulated cultures were required to be lyophilized, 70 ml. spore suspension were diluted with 10 ml. sterile solution (4 %, w/v) of crude undegraded bacterial dextran so that the final dextran concentration was 0.5 % (w/v). This mixture was then filled into sterile ampoules in 2.5 ml. volumes. The ampoules were rapidly frozen by immersion in a mixture of light petroleum and 'Drikold' and dried in a desiccator under a vacuum of 50 μ . Hg for 48 hr. Dry nitrogen was admitted to the desiccator, the cotton-wool plugs pushed down, a second drying effected over P_2O_5 under a vacuum of 50 μ . Hg for 4 days and finally dry nitrogen was again admitted to the desiccator before sealing the ampoules.

RESULTS

Observations of morphology on the urea medium

Flasks of the urea medium were inoculated with a light scrape from a well-sporulated surface culture of a streptomycin-producing strain of *Streptomyces griseus*, or preferably with one ampoule of lyophilized spores (approximately 6×10^7 viable spores) prepared from the glycine medium in the manner described below. The cultures were incubated on a rotary shaker (2 in. throw

at 180 r.p.m.) at 28° for 10 days. Preparations for microscopical examination were made by sampling the cultures at frequent intervals.

The spores germinated within 48 hr. to give a profusely branched mycelium analogous to the 'primary mycelium' of Klieneberger-Nobel. After *c.* 96 hr. this 'primary mycelium' developed deeply staining goblet-shaped bodies, spaced along the hyphae (Pl. 1, fig. 1). Because of the subsequent development of these bodies we refer to them as 'initial cells'. The stimulus for the production of these initial cells appeared to be related to and preceded by anastomosis of the primary mycelium, but we have no unequivocal evidence that hyphal fusion actually occurred.

After *c.* 108 to 124 hr. the initial cells germinated to produce a weakly-branched septate mycelium analogous to the 'secondary mycelium' of Klieneberger-Nobel (Pl. 1, fig. 2). After *c.* 8 days the secondary mycelium rapidly developed chains of closely applied cylindrical spores. We do not know the true nature of these spores; having regard to their method of formation, we term them 'secondary mycelium spores'. Sporulation of the 'secondary mycelium' was not confined to special sporangiophores but proceeded from the tips of the secondary mycelium in an acropetal succession. The secondary mycelium spores were characterized by the fact that they readily separated to lie free in the medium. At this stage the sporulating secondary mycelium was seen to be deeply stained against a faintly stained background of lysing primary mycelium (Pl. 1, fig. 6).

Subsequently, the secondary mycelium also lysed, with the result that at 10 days the secondary mycelium spores lay free in a matrix of medium containing lysed mycelium. At this stage dextran was added and the cultures were lyophilized in 2.5 ml. amounts to provide ampoules containing approximately 6×10^7 viable secondary mycelium spores which on germination (Pl. 1, fig. 5) developed into primary mycelium. Such spores, on inoculation into fresh urea medium, repeated the cycle already described. When the lyophilization step was omitted and the liquid spore suspension was used as inoculum, it was found that mixed cultures of primary and secondary mycelium sometimes resulted. It is believed that this was due to the proliferation of some unlysed secondary mycelium that would have been killed under the normal conditions of lyophilization. In such mixed cultures, no clearly defined sequence of events was observed, presumably owing to the superimposition of two growth cycles which were similar but out of phase.

Some of the urea medium cultures, started from spores, were lyophilized at 96 hr. when initial cells had formed but had not yet germinated. Such initial cell ampoules, when inoculated one to each flask of urea medium, germinated to give substantial amounts of secondary mycelium, which subsequently sporulated to yield typical secondary mycelium spores. It was noted, however, that in cultures started from initial cells, sporulation began after *c.* 3 days and was complete by 5 days, thus preserving the time-scale of the full cycle but eliminating the proliferation stage of primary mycelium.

Several factors influenced the growth cycle in urea medium. Thus the incorporation of increasing amounts of casein hydrolysate (Pronutrin) into the

urea medium progressively favoured the growth of primary mycelium at the expense of initial cells, secondary mycelium and sporulation, until at a concentration of 1 % (w/v) of the hydrolysate, sporulation was almost completely prevented; primary mycelium alone proliferated and finally lysed. An increase in glucose concentration from 5 to 10 g./l. or increased aeration (by increase of the rotary shaker speed from 180 to 220 r.p.m.) similarly favoured primary mycelium and resulted in lower counts of viable secondary mycelium spores.

Observations of morphology on the glycine medium

Flasks of glycine medium were inoculated with a streptomycin-producing strain of *Streptomyces griseus*, in the manner described above for the urea medium. Cultures were incubated on a rotary shaker (2 in. throw and 220 r.p.m.) at 28° for 10 days, and microscopical preparations were made from samples taken at frequent intervals.

The spores germinated within 20 hr. and gave profusely branched primary mycelium. The young primary mycelium stained well and revealed large numbers of bodies which we consider to be nuclei. As the mycelium developed, it gradually lost this characteristic staining reaction until these nuclei could no longer be distinguished. The primary mycelium proliferated for several days. At c. 6–8 days the contents of the primary mycelium rounded-off to produce large numbers of spores. Since these spores were developed directly by the vegetative primary mycelium, and because they appeared to be formed in an intercalary manner, we term them 'chlamydospores'. When mature, these spores tended to remain in long chains of relatively widely spaced spores rather than to become free in the medium (Pl. 1, fig. 7). At this stage the cultures were lyophilized in 2.5 ml. amounts and gave ampoules containing approximately 6×10^7 viable chlamydospores. On germination, these produced typical primary mycelium.

It was observed that chlamydospore formation was depressed by decreased aeration, by incubation on a slower rotary shaker (2 in. throw and 180 r.p.m.) or by the addition of 1 % (w/v) peptone to the medium. With the incorporation of 2 % (w/v) peptone sporulation was completely inhibited and the primary mycelium finally lysed. Proliferation of primary mycelium, culminating in the direct production of large numbers of apparently intercalary spores, was observed in a rather wide range of media, with fairly low concentrations of available nitrogen. Thus, for example, essentially the same process occurred in a medium based on glucose and soya-bean meal.

Media which supported chlamydospore formation when inoculated with primary mycelium also supported the formation of secondary mycelium spores when inoculated with secondary mycelium or lyophilized initial cells. Such media, however, did not provide the stimulus for initial cell formation, and therefore no cycle involving an alternation of two morphologically different types of mycelia was observed.

Observations of morphology on the meat extract + casein-digest medium

Flasks of meat extract + casein-digest medium were inoculated with a streptomycin-producing strain of *Streptomyces griseus* as previously described. The cultures were incubated on a rotary shaker (2 in. throw and 220 r.p.m.) at 28° for 10 days; microscopical preparations were made from samples taken at frequent intervals. The spores developed into primary mycelium which proliferated in much the same manner as that from the glycine medium already described. Growth of primary mycelium was very vigorous but essentially complete within 60 hr. No initial cells or secondary mycelium were formed, the primary mycelium lysing after *c.* 120 hr. Occasionally a few chlamydospores appeared, but sporulation was generally completely suppressed.

DISCUSSION

The nature and origin of the initial cells is of interest, because they appear to arise as a result of hyphal fusion. Some indications as to how they may be formed can be surmised from an examination of their structure and morphology on germination. Typical initial cells of *Streptomyces griseus* (Pl. 1, fig. 1) are formed at intervals along a strand of primary mycelium at points where anastomoses seem to have occurred. Each initial cell usually has at least one filamentous appendage of rather short but variable length. The primary mycelium between initial cells increases in girth, but subsequently loses its contents; at such points the mycelium readily fragments. Fragmentation may or may not precede the germination of the initial cells. Thus free initial cells may be seen to develop into septate secondary mycelium (Pl. 1, figs. 2, 3) or alternatively germination may occur *in situ* (Pl. 1, fig. 4). The filamentous appendages are usually longest when the initial cells are first recognized (Pl. 1, fig. 1), and remain as mere fragments by the time germination of the initial cells is under way (Pl. 1, fig. 3). It may be that these appendages are the remains of primary mycelium hyphae that have taken part in anastomoses and then become moribund. Rigid proof of anastomosis before initial cell formation, however, is unlikely to be obtained from visual observations alone; genetical evidence would probably be required.

There is no proof that the morphological forms observed in our submerged cultures in urea medium are the same as those found by Klieneberger-Nobel for surface cultures, but there is a marked similarity. These morphological forms cannot be explained as artefacts, since they only occur under certain cultural conditions. Moreover, free initial cells have been observed in all stages of germination. It may therefore be concluded that *Streptomyces griseus* exhibits an alternation of morphological generations in submerged culture in urea medium similar to that described by Klieneberger-Nobel for surface cultures, primary mycelium alternating with secondary mycelium and the two generations being separated by an initial cell stage. The secondary mycelium sporulates to yield secondary mycelium spores. The stimulus for initial cell formation is unknown, but the requisite conditions in submerged

culture are believed to be rather narrow and to be conditioned by aeration and the type of nitrogen supply. Media which do not support initial cell formation may permit the development of intercalary chlamydospores directly from primary mycelium.

The use of dextran for lyophilization was developed jointly with Miss J. O. R. Day, while the lyophilization procedure was determined in co-operation with Dr N. Blakebrough and Mr H. M. S. Kitching. The photomicrographs were obtained by Mrs K. M. King, Dr P. B. Dickenson and Mr R. McWilliam. Valuable technical assistance was afforded by Miss E. Theakston throughout.

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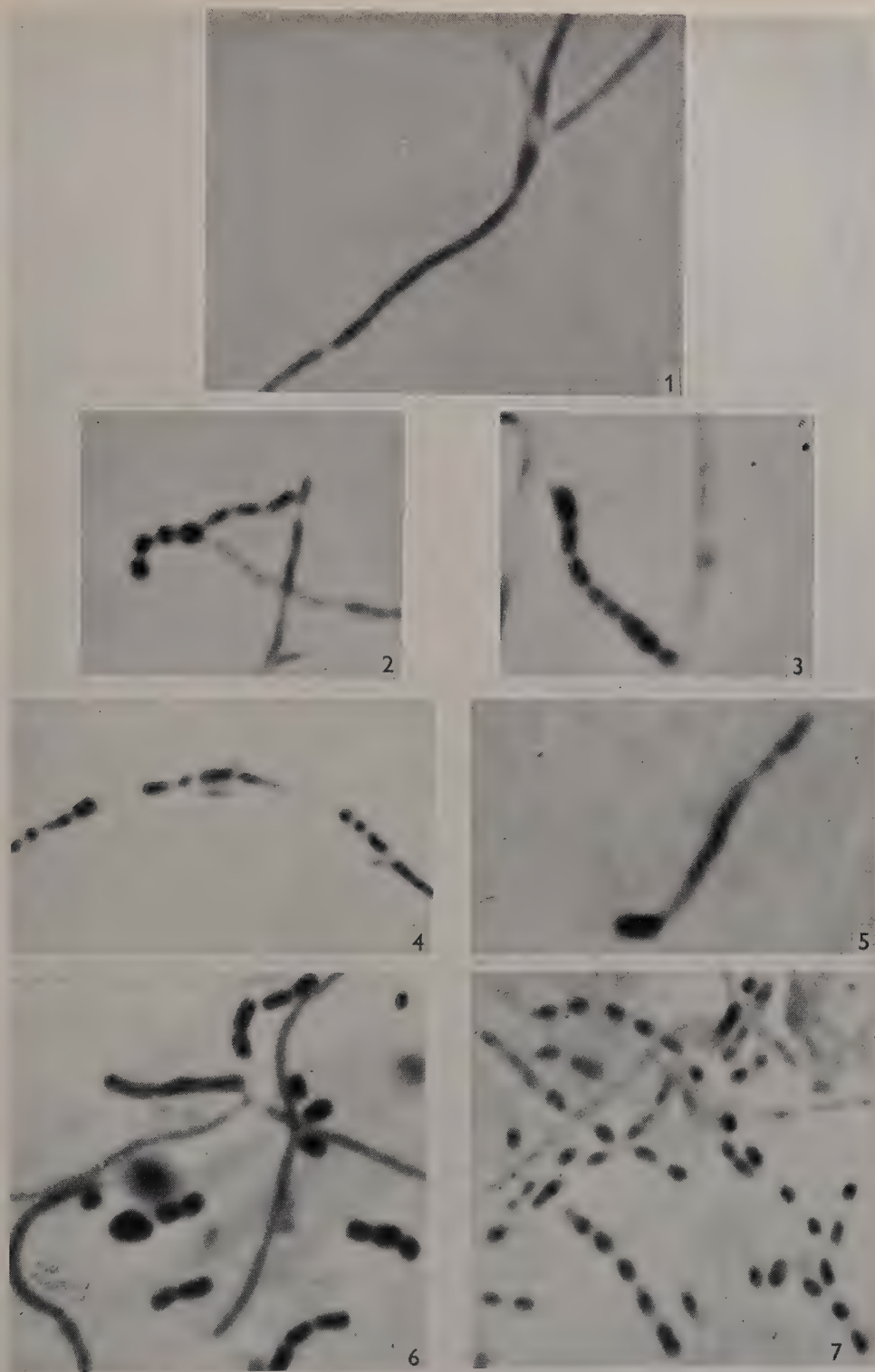
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EXPLANATION OF PLATE

All figures show cultures of a streptomycin-producing strain of *Streptomyces griseus* stained with basic fuchsin; $\times 6000$.

- Fig. 1. Young initial cells.
- Fig. 2. Free initial cells and young septate secondary mycelium derived from them on germination.
- Fig. 3. Free initial cells and secondary mycelium at a later stage than that shown in fig. 2.
- Fig. 4. Initial cells which have germinated to form secondary mycelium before separation from the site of their formation in primary mycelium.
- Fig. 5. Germ tubes of primary mycelium from secondary mycelium spores.
- Fig. 6. Typical appearance of a sporulated culture in the urea medium showing secondary mycelium spores.
- Fig. 7. Typical appearance of a sporulated culture in the glycine medium showing chlamydospores.

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G. D. WILKIN & A. RHODES—MORPHOLOGY OF *S. GRISEUS* IN SUBMERGED CULTURE. PLATE 1

(Facing p. 264)

The Inheritance of Differences in Growth Rate in *Escherichia coli*

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SUMMARY: In cultures from single cells of *Escherichia coli* grown under anaerobic conditions there are variations in rate of growth of the individual colonies as measured by their diameter after a short incubation. When small and large colonies are selected the populations derived from them inherit the tendency to produce correspondingly smaller or larger colonies than the mean of those produced by the parent strain. When the growth conditions are altered then the selected character will be lost.

It was noticed that in *Escherichia coli*, strain Brent, the colonies grew at different rates under anaerobic conditions in a de Fonbrune oil chamber. This was a character that could be studied in much the same way as size in higher plants (cf. East, 1915) and it seemed possible that fast- and slow-growing strains could be selected and that it might be shown that the rate of growth was an inherited characteristic.

At first accurate histograms were not obtained since the random distribution of an implant on a solid medium results in some of the colonies being derived from more than one parent cell. Also, for the test to be convincing the parent strain and the various daughter strains selected from it should be cultivated on the same batch of medium under identical conditions. Fortunately a technique has been found which enables as many as 500 single cells from five strains to be dealt with in the same oil chamber on samples of the same medium.

METHOD

A single cell was picked by means of the micromanipulator pipette and transferred to a drop of Hartley's digest broth where it was incubated for 3 hr. at 37°. The drop of suspension was withdrawn from the oil chamber and transferred into 10 ml. of broth and there incubated for a further 3 hr. at 37° with continuous agitation. This suspension was retained and stored at 4° and formed the parent strain, suspension no. 1.

Solid medium was prepared from the same broth with 2% Kobe no. 1 shred agar. A layer of the melted agar was poured on a sterilized glass slide; after cooling, the agar was trimmed and cut up into squares 0.5 cm. across and one of these was transferred to the coverslip of an oil-chamber with a scalpel. A loopful of the stored culture was spread on this square, the coverslip was inverted and the chamber filled and incubated at 37° for 3 hr.

After incubation the colonies were inspected and the smallest was picked into a drop of broth. A segment of one of the largest colonies, containing

about the same number of cells, was similarly treated. These two new suspensions, the small no. 2 and the large no. 3, were transferred, without incubation in the chamber, into tubes of broth, incubated for 3 hr. at 37° and then stored with the parent suspension at 4°. In turn, samples of these two suspensions were spread on agar blocks and incubated and the smallest colony from no. 2 and a segment of the largest from no. 3 taken to form another pair, no. 4 and no. 5 respectively.

On the following day the three stored cultures were taken and 0.1 ml. of each was incubated with 9 ml. of broth for 2 hr. at 37°. The oil chamber was prepared with five blocks of agar arranged so that they were equidistant from the edge of the coverslip. The distance from the edge of the coverslip is important since, when the blocks were not separated from the air by a margin of 0.3 mm. or more, it was found that colonies developing at the exposed edge had an advantage and uniform results were not obtained.

A loopful of suspension no. 1 was now spread on the first block; similarly, suspensions nos. 2 and 3 were given a block each. Suspensions nos. 4 and 5 had been retained in the oil chamber at 4° and as much as possible of each of these was transferred to the appropriate remaining block without further subculture, the coverslip was inverted and the oil chamber filled.

While it is difficult by micromanipulator to plant out large numbers of single cells, it proved relatively easy to thin out the implant (made with a loop in the ordinary way) by removing the excess cells with a micropipette so that there was not more than one cell to each field of the microscope at a magnification of 240.

The manipulation took place at room temperature and it was possible to obtain the necessary 100 single cells on each of the five blocks before their first division had been completed. When 100 cells had been so isolated on one block the area was marked and any remaining cells left unthinned.

The preparation was now incubated at 37° for 3 hr., cooled in the refrigerator to stop further growth and the colony diameters measured using an eyepiece micrometer. The results are expressed in Fig. 1 as direct measurements of colony diameter.

RESULTS

The histograms of the subcultures show that from the single cell giving the first culture it is possible to select out populations which grow either more or less rapidly than the parent strain in the particular environment provided. There was no detectable delay in growth starting in the slow-growing culture. In culture 4 the small colony strain gave rise to cells which were unable to divide and many of which gave the long forms previously reported with this organism (Hughes, 1953).

Once small colonies have been obtained, with re-selection a strain can be kept which produces uniformly small colonies under the conditions in the oil chamber. When this strain was grown aerobically in broth the turbidity reached was not obviously different from that of the parent or the large colony strains. When, however, it was returned to the oil chamber it showed the small

colony character. Three or more overnight subcultures in aerobic broth without re-selection, however, were followed by a blurring of the pattern and the strains approximated progressively to the parent.

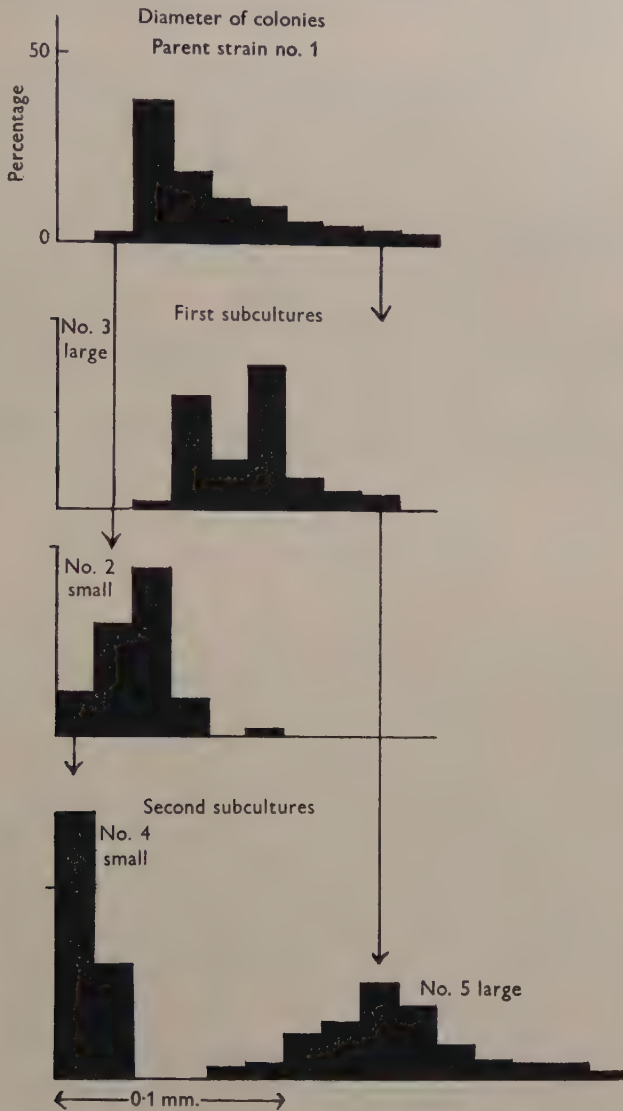


Fig. 1. Histogram showing rates of growth of colonies from single cells of *Escherichia coli* grown under anaerobic conditions. Measurements of colony diameter made after 3 hr. incubation at 37°. Re-selection of the subcultures indicated by arrows.

DISCUSSION

It would appear that variation takes place in cultures of organisms derived from a single cell. This variation is apparently not due to minor differences in the environment since in these experiments such differences were reduced to

a minimum. The populations breed true for just as long as no other selection by a different culture medium or atmospheric condition is applied to them. It is not possible at this stage to indicate what it is in the large colony strain that gives it an advantage over the small. Both seem to grow equally well in ordinary aerobic culture.

The general results agree well with those obtained by Rogers (1953). When he was selecting strains for hyaluronidase production, he also used an entire population but felt that in this there was a relatively small number of discrete populations differing in their ability to form enzyme. Here it is not obvious that any sharp lines can be drawn between the various populations, though further experience may show that the differences between them can be broken down into discrete steps.

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The Differences in Antibiotic Sensitivity of Closely Related Single Cells of *Proteus vulgaris*

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SUMMARY: Evidence is offered that when a single bacterial cell divides the daughter cells are frequently unlike one another. This can be demonstrated by separating cells, allowing them to divide and then transferring individuals derived from them to agar containing a suitable concentration of antibiotic. Differences between single cells can be tested by four criteria: (1) division; (2) growth without division; (3) ability to resume division when transferred back to normal broth; (4) failure to lyse.

Previous results on the distribution of antibiotic sensitivity in cultures from single cells show that a wide range of differences is present (Hughes, 1952; Eagle, Fleischmann & Levy, 1952). This would be explained if either mutation were very frequent or if changes appeared at each division giving two daughter cells which might differ appreciably from each other. Evidence that two cells with different characteristics could arise from the division of a single parent cell has already been presented (Hughes, 1953*a*). Then it was shown that under anaerobic conditions on nutrient agar single cells of coliform organisms divided to give one cell which was normal and established a colony, and one cell which was unable to divide and gave rise to a long form.

It had also been noticed that when a culture of *Proteus vulgaris* grown from a single cell was exposed to penicillin, the appearances of the cells differed according to the concentration of the antibiotic (Hughes, 1953*b*). Also, at any one concentration the effect on the individual cells differed so that some were able to multiply while others were unable to divide or were lysed (Table 1). One typical field from such a culture is shown in Pl. 1, fig. 1. Of three organisms all derived from a 3 hr. subculture one is undergoing autolysis, one has grown but is unable to divide, while one has already divided into four, of which three are showing early swellings while the fourth, though large, shows no definite abnormality.

A method for examining individual cells of *Proteus vulgaris* for resistance or susceptibility based on this type of observation was elaborated.

METHODS

The experiments were carried out in the oil-chamber of a de Fonbrune micro-manipulator and observed by phase-contrast microscopy.

A batch of Hartley's digest broth was prepared, filtered to free it from any dead organisms, measured into bottles and used throughout the experiments. From the same batch of broth 2% nutrient agar was also prepared

Table 1. *Effect of variation of concentration of penicillin on the morphology and viability of individual cells of Proteus vulgaris*

Morphology of cells	Concentration of penicillin in units/ml.				
	0	2	3	4	5
Normal	93	55	20	0	0
Abnormal dividing	6	30	76	36	17
Abnormal not dividing	0	12	4	46	77
Lysed	0	0	0	18	6

and dispensed into screw-capped bottles each containing 2.0 ml. For use one bottle of agar was melted and an equal volume of penicillin dissolved in broth added to give the required final concentration in 1% agar. A large drop of the penicillin agar was then poured on to a sterile slide and allowed to set. A number of blocks 1 × 1.5 mm. in size were cut from this and arranged on

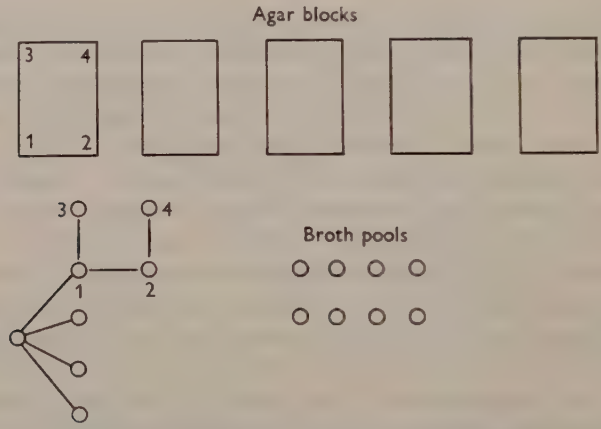


Fig. 1. Suitable arrangement for the study of the sensitivity of single cells to antibiotics.

the coverslip, together with a pool of sterile broth and a separate drop of a 3 hr. culture of the stock organisms in broth. The coverslip was inverted and the chamber filled with oil. Next, a pattern of drops of broth was made with the micropipette, arranged as shown in Fig. 1.

A single cell was taken into the first drop and incubated either at room temperature or at 37° until it had divided into four or more. Each of these cells was placed in a separate drop and allowed to divide to give 4 or more cells which were again separated. At this stage differences in growth rates were apparent and during the time that one of the small cultures took to become four cells others had reached totals of up to sixteen. Each of these small groups was allotted an agar block containing penicillin and each cell was placed at a marked position on it so that it could be identified, observed and rescued at the appropriate time.

Since it might have been thought possible that organisms could influence one another genetically or otherwise by sharing a block, the experiment was reduced in scale and the patterns modified by providing a separate block of agar for each cell. When this was done no differences were detected and it appeared to be an unnecessary complication.

The preparation was now incubated at 37° for 2 hr. when the blocks contained 10 u. penicillin/ml., or overnight when lower concentrations were used. With other strains the concentration and time would be different. Four tests can now be applied to the individual cell; (1) division; (2) growth without division; (3) ability to resume growth when returned to normal broth; (4) failure to lyse. Using these criteria the sensitivity of individual cells was examined.

After incubation the cells, whether apparently affected by the exposure to penicillin or not, were transferred to drops of normal broth and the preparation reincubated. Cells which did not divide were assumed to be dead or dying. A few of them, though obviously motile, were dying, for division was not resumed; it was found that these all lysed within 48 hr., like all those which failed to divide. Since the large forms produced on penicillin consist of more than one cellular unit, it is possible for part to be dead and the remainder living; such an organism was classed as living but its occurrence emphasizes that differences are present before division has taken place.

RESULTS

Even without penicillin being present, a small number of cells from most strains will be adversely affected. Controls with nutrient agar free of penicillin were examined. These showed up to 1 % of long forms which were unable to divide.

A typical experiment using 10 u. penicillin/ml. in the agar is shown in Fig. 2. The microculture consisted of a clone of sixteen cells, of which nine were taken to establish small groups and details of these are given; it was impossible in the time to handle the remainder. Cell 1 appears the most sensitive, only one daughter cell was able to grow at all, none divided and all four were dead within 2 hr. Cell 3 gave the most highly resistant group with three viable cells, all cells survived for some time, grew into long forms and three divided or completed division. Cell 5 was intermediate between the other two and was representative of the remaining six groups. It may be significant that of the six viable cells demonstrated by transferring to normal broth, three were in a single group.

Fig. 3 gives the results of overnight cultures of eight microcolonies on weaker penicillin. Here one cell was taken and allowed to develop into eight which were isolated from each other and allowed to continue growing to give the eight related groups. Differences in growth rates were now apparent, the numbers of cells produced in the same time ranging from three to eight. These were explanted on 7.5 u./ml. penicillin agar. At this concentration with this preparation long swollen forms were not produced and the cells either lysed or formed small colonies during the night.

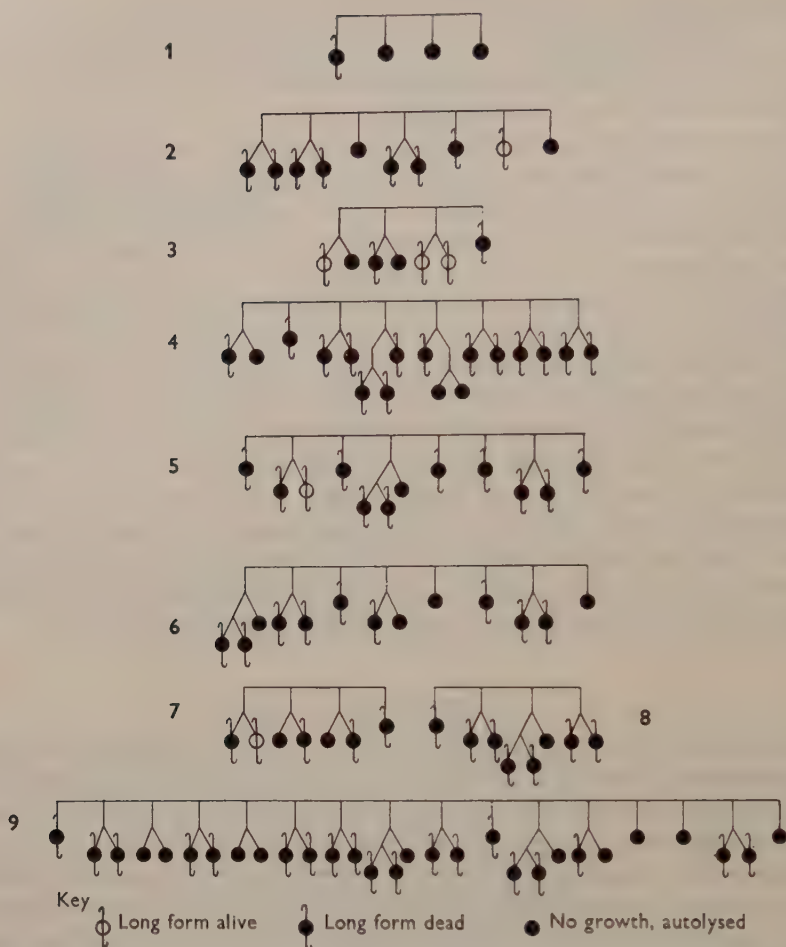


Fig. 2. The effect of 10 u. penicillin/ml. on the individual cells of nine microcultures, grown from nine members of a clone of sixteen cells.

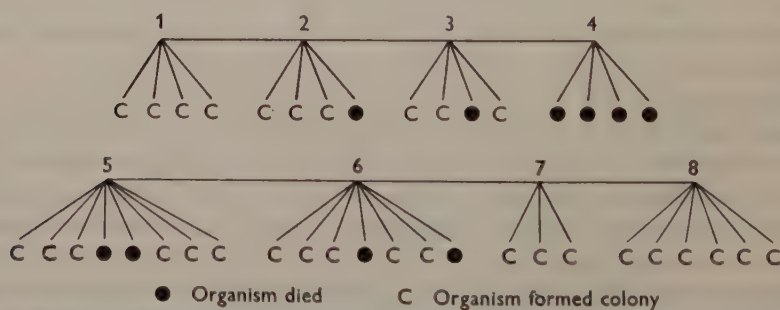


Fig. 3. Diagram showing fate of eight microcolonies derived from the same single cell when plated out on 7.5 u. penicillin/ml. agar.

Here, as in the last experiment, sensitivity is not distributed evenly throughout the groups; for instance, the whole of group 4 lysed.

Although this work was done with penicillin and *Proteus vulgaris*, experiments were also made with coliforms, and with other antibiotics such as streptomycin and terramycin, which gave similar results.

DISCUSSION

The type of variation illustrated by these experiments has been little studied. There is no reason to suppose that transformation, transduction or any form of genetic recombination is taking place. If we regard the varying sensitivity of closely related cells as due to mutations, we must allow a very high rate of mutation, of the order of one in two divisions. The fact that the more closely related the cells are the more likely they are to share either sensitivity or resistance suggests that these characteristics are determined before contact with the antibiotic though they can only be detected in its presence. It may be necessary to test at each division, rather than allowing small groups to develop, in order to establish this. One explanation would be that before each division the factors determining hereditary characters, whether equivalent to the genes of higher plants or not, are redistributed in such a way that their spatial relationship is altered. During such a process nothing would be lost by the cells; no permanent changes would take place so that if the more sensitive cells were rescued from the adverse influences in time the cultures derived from them would contain similar elements to the parent strain, though the proportion of sensitive and resistant cells would have altered. Were the loss of a hereditary element responsible for either sensitivity or resistance, then the sensitive or resistant cell would produce only progeny of the same type, unless there were introduction of fresh genetic material; these experiments were designed to avoid any possibility of the latter.

To keep these strains constant a selection pressure must be maintained for a considerable time. It has proved possible by selection of single cells from cultures exposed to 5 u. penicillin/ml. as in experiments described previously, choosing those that were most normal in appearance, subculturing from these and repeating the process, to increase the average resistance of a culture, but after twenty-six such subcultures an increase in tolerance from 5 to 10 u. penicillin/ml. was all that had been obtained.

In previous work in which resistant strains have been bred out from populations, no evidence was available of the frequency with which more sensitive cells appeared in the cultures, since most methods used were designed to kill off all but the most resistant variants. Rowley (1952) has shown that sensitive mutants appear in irradiated cultures but he was dealing with induced mutations. The 'family tree' type of experiment used here establishes that there are differences between sister cells. Cooper at the April 1954 meeting of the Society for General Microbiology suggested that the stage of growth at the time of exposure may be the determining factor in a cell's survival. Growth rates, even of members of a microcolony, are certainly different and the

individual cells will be at different stages when planted out. Nevertheless, some of them divide on the penicillin agar and then it will be impossible for the daughter cells to be in different stages of growth, yet one may survive while the other dies. Unequal distribution of hypothetical cytoplasmic elements between the two cannot be entirely excluded.

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EXPLANATION OF PLATE

Fig. 1. Variation in effect of penicillin on cells of *Proteus vulgaris* derived from the same single cell.

(Received 11 October 1954)



W. HOWARD HUGHES—DIFFERENCES IN RELATED SINGLE CELLS. PLATE 1

(Facing p 274)

The Role of Divalent Cations in the Multiplication of Staphylococcal Bacteriophages

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SUMMARY: The staphylococcal typing phages require divalent cations for a stage in phage growth which is probably that of penetration. When phage is adsorbed to the cell surface of sensitive cocci and a chelating agent is added, there is a short period during which the phage particle may be inactivated and its lethal effect on the coccus prevented. Cocci attacked by phages of serological group A show penetration of the phages in distilled water + CaCl_2 , or + MgCl_2 , or to a less extent + SrCl_2 . The phages of group B are unable to penetrate in presence of SrCl_2 and in some cases MgCl_2 is only partially active as compared with CaCl_2 . Ca ions appear to be required specifically at a late stage in phage synthesis since the average yield of phage/coccus increases with increasing concentration of CaCl_2 ; the addition of a chelating agent towards the end of the latent period depresses the yield of phage.

Some bacteriophages require Ca ions either for adsorption or during phage multiplication. With coliphage T5 (Adams, 1949; Luria & Steiner, 1954), a typhoid phage (Fildes, Kay & Joklik, 1953) and lactic streptococcus phages (Potter & Nelson, 1953) Ca is not necessary for adsorption but apparently acts at an early stage in phage synthesis. Delbrück (1948) and Beumer & Beumer-Jochmans (1951) described two phages (a mutant of coliphage T4 and a dysentery phage) which apparently required Ca for adsorption. However, Fildes (1953) pointed out that in these cases the activity of alternative divalent cations was not investigated and it is therefore uncertain whether or not the Ca requirements were absolute.

Puck (1953), with coliphages of the T series, produced evidence that there were a number of stages in the process of phage invasion. He suggested that the first step of adsorption involved electrostatic forces which were supplied by cations of the medium and which permitted reversible attachment of phage to cell surface. The next step was the separation of the protein and deoxyribonucleic acid components of the phage; this is quickly followed by a process leading to cell killing, and the penetration of deoxyribonucleic acid into the cell and the initiation of phage synthesis in lytic systems. The process of penetration is probably enzymic in nature. The staphylococcal typing phages (Rountree, 1951) require divalent cations for adsorption; this process is inhibited by Na citrate. Each phage has characteristic cation requirements which are related to the phage rather than to the host cell surface. The present paper deals with the role of divalent cations in the different stages of multiplication of staphylococcal phage which follow the first step of adsorption.

METHODS

The typing phages of Wilson & Atkinson (1945) and their respective propagating strains were used. The methods of phage estimation, preparation of glassware and of salt solutions have been previously described (Rountree, 1951). In general, phage counts were made in quadruplicate. Since no chemically defined medium which regularly supported the growth of the phages was available, glucose (0.15 %, w/v) veal broth was used as basal medium which was supplemented with various cations as required. The disodium salt of ethylene diaminetetra-acetic acid (EDTAA) (supplied by Dr F. Dwyer, University of Sydney) was used as a chelating agent. Na citrate was unsuitable for this purpose since in the concentrations necessary for effective chelation it inactivated many of the phages when they were in the free state.

The agar nutrient broth medium used for plaque counting had 0.1 % (w/v) CaCl_2 added to it.

RESULTS

One-step growth curves

Knowledge of the lengths of the minimum latent period and of the one-step growth curve and average burst size were required for the various different phages. One large batch of broth was prepared which supported the growth of all the phages.

The appropriate propagating strain of staphylococcus was grown in broth with aeration in a water bath at 37° until the total count (determined turbidometrically) reached 1×10^8 cells/ml. Sufficient phage was then added to give 3–4 particles/cell. After 10 min. of adsorption the infected cells were so diluted in warmed broth that on plating 0.01 ml. samples 1–3 plaques were obtained. Incubation was continued at 37°, samples being removed for plating at 5 min. intervals. The end of the latent period was marked by an increase in the plaque count. If all cells had been infected, the end of the period of phage release was followed by a steady count. The average burst size was obtained by dividing the final phage count calculated in terms of the original volume of infected culture by the number of infected cells.

The phages showed fairly wide differences in the length of the minimum latent period; this ranged from 35 min. for phage 3B to 60 min. for phage 42B (Table 1). Most of the phages, however, gave their first release of phage between 40 and 50 min. The end of phage release ranged from 50 to 75 min. In this particular batch of broth the average yield of phage particles per cell ranged from 12 for phage 42B to 168 for 47D. Replicate estimations of the average burst size of different phages agreed well, although exact reproduction of results from day to day was not always possible. This was thought to be due chiefly to variability in cell sensitivity. Variation in average burst size also occurred with different batches of broth.

Table 1. *Times of the minimum latent period and the end of phage release of staphylococcal phages grown in broth*

Latent period (min.)	Phages	End of phage release (min.)	Phages
35-40	3B, 44A	50-55	47D, 51, 52A
40-45	3C, 6, 7, 29, 29A, 31A, 31B, 42C, 44, 47A, 47D, 51, 52, 52A	55-60	3C, 6, 7, 29A, 31A, 44A, 47A, 52
		60-65	3B, 29, 42C, 42D, 44, 47, 47B
45-50	3A, 31, 42D, 42E, 47, 47B, 47C	65-70	31, 31B, 42B, 42E
55-60	42B	70-75	3A, 47C

Effect of divalent cations on the average burst size

Williams Smith (1948) observed that the addition of CaCl_2 to broth in which staphylococcal phages could not be propagated rendered the medium adequate. This stimulatory effect of CaCl_2 might be due solely to its effect on phage adsorption but it seems probable that Ca ions were required for a later step or steps in phage synthesis. In the present investigations, one particular batch of broth which appeared to be deficient in calcium, since some phages failed to multiply in it but added CaCl_2 made it adequate for growth, was used to examine the divalent cation requirements of the phages. Experiments were so arranged that growth tubes contained two to three infected cells/0.01 ml. during the latent period and so yielded countable numbers of plaques when plated directly at the end of the rise in free phage. To a series of $6 \times \frac{3}{4}$ in. test tubes (wide tubes to ensure adequate aeration) was added 0.1 ml. of solutions of either CaCl_2 , MgCl_2 or $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$ to give final salt concentrations of 20, 50, 100 and 200 $\mu\text{g./ml.}$ Samples of young aerated broth culture (0.8 ml.) of the appropriate staphylococcus were added to each of the tubes. These were placed in a water-bath at 37° and then received 0.1 ml. of phage suspension (diluted in broth). Control tubes of cells + phage without added salt, and of the phage in broth + salts, were included in each test. Samples were plated during the latent period to determine the number of infected cells and at the end of 70 or 80 min., depending on the growth curve of the particular phage being examined. The average burst size was calculated by dividing the final phage count by the count during the latent period.

All the phages examined showed an increase of the average burst size on addition of CaCl_2 , but there were differences in the behaviour of different phages which could be correlated with their serological types. Table 2 shows the average burst size obtained for twenty different phages belonging to serological groups A and B (Rountree, 1949). The group A phages were able to establish infection in the basal broth alone and their average burst sizes were, in most cases, of a reasonably high order; however, the addition of CaCl_2 increased their burst sizes. The amounts of CaCl_2 which gave optimal phage production varied and was 20 $\mu\text{g./ml.}$ with phages 3C, 7 and 51, 50 $\mu\text{g./ml.}$

with phage 6, and 100 $\mu\text{g.}/\text{ml.}$ with the remainder. When MgCl_2 or SrCl_2 was substituted for CaCl_2 no clear-cut evidence of increased yield was obtained. Possible exceptions were phage 6 in which an average burst size of 64 was produced in the presence of 200 $\mu\text{g.}$ $\text{MgCl}_2/\text{ml.}$ as compared with 44 in the basal broth, and phage 7 which gave average burst sizes of 16 and 21 in 100 and 200 $\mu\text{g.}$ $\text{SrCl}_2/\text{ml.}$ respectively, as compared with 7 in broth alone.

Table 2. *Effect of added divalent cations on the average burst size in broth of staphylococcal phages*

The phages in the upper half of the table (3A to 51 inclusive) belong to serological group A; those in the lower half (29 to 52A inclusive) to serological group B. Average burst size obtained by plating at 70–80 min. after infection, 0 = disappearance of phage, 1 = adsorption but no multiplication.

Phage	Composition of broth + salts mixtures												
	Broth alone	Broth + CaCl_2 $\mu\text{g. CaCl}_2/\text{ml.}$				Broth + MgCl_2 $\mu\text{g. MgCl}_2/\text{ml.}$				Broth + SrCl_2 $\mu\text{g. SrCl}_2/\text{ml.}$			
		20	50	100	200	20	50	100	200	20	50	100	200
		Average burst size											
3A	24	28	68	85	77	35	28	45	47	12	14	14	12
3B	111	116	126	152	140	93	113	80	95	108	118	100	95
3C	12	27	26	22	29	20	18	20	20	20	17	15	16
6	44	66	122	90	106	45	54	46	64	52	42	54	46
7	7	44	47	42	33	9	12	13	10	10	10	16	21
42E	3	3	12	26	60	2	3	2	2	2	2	2	3
47	20	43	56	100	68	24	22	20	12	18	21	19	17
47A	29	24	50	61	63	16	20	21	22	23	28	22	37
51	75	101	105	110	111	72	75	85	83	85	81	85	95
29	2	9	20	24	10	2	3	5	7	3	3	3	3
29A	6	41	92	91	30	6	6	15	29	4	14	14	10
31	1	18	25	26	50	3	4	7	14	3	3	5	10
31A	1	9	20	19	15	5	2	4	9	1	3	2	2
31B	10	20	45	71	102	3	3	6	12	2	4	8	9
42C	0	0	14	18	15	0	0	0	0	0	0	0	0
42D	0	0	31	52	24	0	0	0	0	0	0	0	0
44	1	33	44	85	65	0	0	0	0	0	0	0	0
44A	3	16	37	60	61	4	8	9	8	4	4	6	8
52	0	5	44	63	60	0	0	0	0	0	0	0	0
52A	12	36	57	95	91	7	16	24	41	15	11	24	26

Many of the group B phages did not grow in the basal broth. Phages 42C, 42D and 52 disappeared partly on adsorption, but chiefly during the latent period; and the addition of at least 50 $\mu\text{g.}$ $\text{CaCl}_2/\text{ml.}$ was necessary before any phage multiplication took place. With these phages and with phage 44, the addition of MgCl_2 or SrCl_2 to broth did not prevent this disappearance of phage. Other phages (e.g. 31 and 31A) established infection in broth alone but the infected cells liberated either very little phage or none until plated. All the phages gave higher average burst sizes in the presence of CaCl_2 ; 100 $\mu\text{g.}$ $\text{CaCl}_2/\text{ml.}$ was sufficient for maximal stimulation, with the exception of phages 31 and 31B which required 200 $\mu\text{g.}$ $\text{CaCl}_2/\text{ml.}$ Some phages (e.g. 29A, 31, 31A, 31B, 44A and 52A) gave slight increases in average burst size when MgCl_2

or SrCl_2 was substituted for CaCl_2 . In no case, however, did the average burst size approach that obtained with CaCl_2 . It was concluded that these other cations were at best poor substitutes for Ca ions. Since the previous work had shown that in most cases Mg ion was as effective as Ca ion in the first step of phage adsorption, it was apparent that the stimulatory effect of Ca ion was concerned with some later stage of phage growth.

Divalent cation activity early in the latent period

The phages 3A and 29, representative of groups A and B, were used for further study of the stage at which Ca ions were required, EDTAA (0.1 %, w/v) being used as a chelating agent at various times in the latent period. This concentration of the chelating agent had no effect on the infectivity of the free phages in broth at 37° during 60 min. (Table 3). The effect of the chelating

Table 3. *Stability of phages 3A and 29 in broth + 0.1 % ethylene diamino-tetra-acetic acid (EDTAA)*

Time of sampling (min.)	Phage 3A		Phage 29	
	In broth	Broth + EDTAA	In broth	Broth + EDTAA
	Phage count ($\times 10^{-3}/\text{ml.}$)			
0	19	18	145	149
60	21	18	152	141

agent on the growth of uninfected cultures of the propagating strains of staphylococci PS3A and 29 was examined. EDTAA (0.1 %, w/v) was added to 3 hr. old broth cultures aerated at 37°; turbidities were determined at intervals thereafter. Figs. 1 and 2 show that the chelating agent had no effect on the growth rate until 50–60 min. had elapsed. Since in the experiments to be described the chelating agent was not in contact with the infected cells for more than 60 min., and often only for 5–10 min., it was concluded that the effects obtained might be attributed to action on the host-phage complex rather than on the cells alone. When EDTAA was added to cells in broth before adding either phage, it completely inhibited adsorption, and all the phage added was recovered in supernatants when the cell + phage mixtures were centrifuged.

Phage 3A. A young broth culture of staphylococcus PS3A was infected with sufficient phage to give about 100–150 plaques/0.01 ml. during the latent period. At 1, 2, 4, 6 and 20 min. after infection, 1 ml. volumes were removed to tubes containing 0.1 ml. 1 % EDTAA and centrifuged (angle centrifuge). After plating samples of the supernatants to estimate unadsorbed phage, the supernatant fluids were removed, the cells resuspended in 1 ml. broth and held in an ice + water bath until plated, as a precaution against phage release before plating. Table 4 shows that, compared with the control which was centrifuged at 6 min., EDTAA added at 1–6 min. markedly decreased the number of infected cells which could be recovered. The maximal effect occurred when the

chelating agent was added at 2 min; 89 % of the adsorbed phage was then inactivated. When EDTAA was added at 20 min. there was little or no inactivation of phage.

Subsequent experiments confirmed that the period 2-3 min. after addition of the phage was the critical time at which the chelating agent had its maximal inactivating effect. Ten minutes after infection the phage+host complex

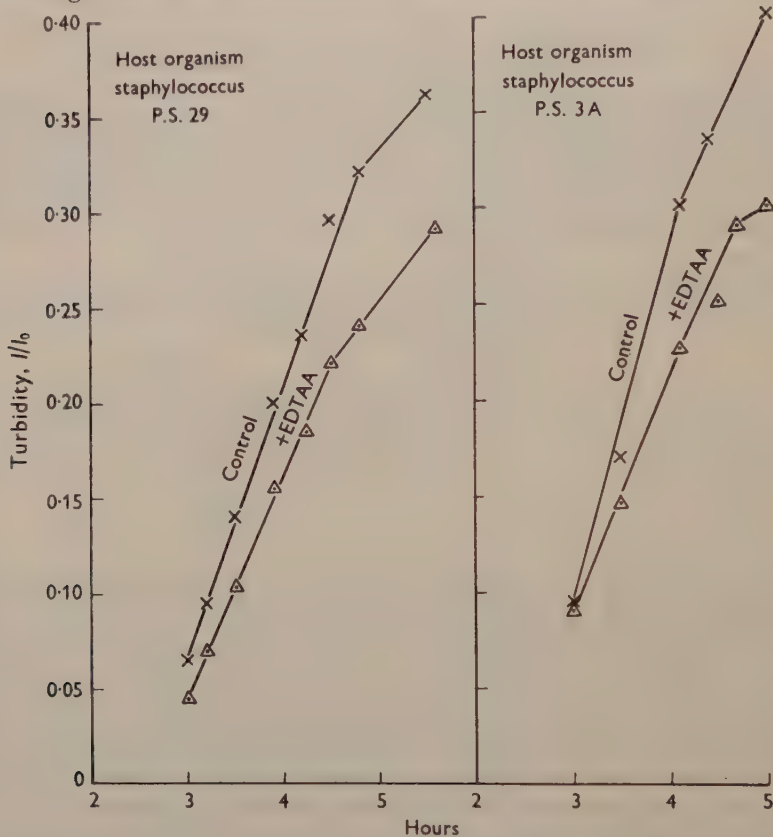


Fig. 1.

Fig. 2.

Figs. 1 and 2. The effect of EDTAA (0.1 %, w/v) added at 3 hr. on the growth in broth of propagating strains of staphylococci no. 29 and 3A.

reached a stage where the removal of divalent cations had no effect on the ability of the infected cells to register as infective centres when plated. This critical period might be called the period of vulnerability. The recovery of some infective centres when EDTAA was added at 1-4 min. indicated that already at these times a few particles had passed through this vulnerable period. The period of vulnerability for any individual particle might be of very short duration and the later in the latent period that the chelating agent was added, the greater the number of particles that would have been adsorbed and have passed this point (see Table 4). In these experiments, the chelating agent was in contact with the cells for at least 8 min. before it was removed in the

supernatant fluid on centrifuging. In order to ascertain the minimum length of time of contact necessary for inactivation of the adsorbed phage, the action of the chelating agent was halted by dilution, for 0.01 % EDTAA had no inactivating effect. The cells received ten times the previous amount of phage, EDTAA was added 2 min. later and, at intervals, 0.1 ml. samples were diluted in 0.9 ml. broth in tubes held in an ice + water bath to prevent further phage

Table 4. *Effect of time of addition of 0.1 % EDTAA on recovery of adsorbed phage from cells infected with phage 3A*

Tube	EDTAA added at (min.)	Phage added (a)	Phage in supernatant (b)	Phage adsorbed (a-b)	Phage recovered in cells	% of adsorbed phage recovered
1	Control no EDTAA	145	45	100	100	100
2	1	145	94	51	14	27
3	2	145	80	65	7	11
4	4	145	68	77	32	42
5	6	145	50	95	49	53
6	20	145	13	132	118	90

Phage/0.01 ml.; mean of 4 replicates

Table 5. *The effect of dilution into broth of phage 3A + cell mixtures to which 0.1 % ethylene diamino tetra-acetic acid (EDTAA) was added*

No EDTAA was added to tube 1; EDTAA was added to tubes 2-6 at 2 min. after the addition of phage.

Tube	Time of dilution	Time of contact with EDTAA	Phage adsorbed/0.01 ml.	Phage in cells	Phage inactivated
1	1 min. 55 sec.	—	82	82	0
2	2 min. 50 sec.	50 sec.	82	42	40
3	3 min.	60 sec.	82	46	36
4	3 min. 30 sec.	90 sec.	82	41	41
5	4 min.	120 sec.	82	42	40
6	*4 min. 10 sec.	> 8 min.	82	32	50

* Tube 6 diluted into broth + EDTAA

Phage/0.01 ml.; mean of 4 replicates

adsorption. The samples after chilling were centrifuged and plated as before. Table 5 shows that approximately 50 % of the phage adsorbed to the cells at 2 min. was inactivated by 50 sec. contact with the chelating agent. Tube 6, in which the mixture was diluted in broth + 0.1 % EDTAA, served as a control; it indicated that there was little if any reversal of the phage inactivation on dilution into broth. A very short period of exposure to the chelating agent was therefore sufficient for irreversible inactivation of adsorbed phage.

Infection with lytic phage normally results in death of the cell; EDTAA (0.1 %) added to cells 3 min. after infection protected at least half the cells from this lethal action. Table 6 shows the results of two experiments with different phage/cell ratios in which viable cell counts were made 10 min. after infection, the centrifuged infected cells being diluted in broth which contained phage antiserum.

Table 6. *The protection of cells infected with phage 3A by the addition of ethylene diamino tetra-acetic acid (EDTAA) 3 min. after infection*

Treatment	Cells/ml.	Phage/cell adsorbed	Viable cells/ml.	Cells killed (%)
0.1 % EDTAA	5×10^7	3	2.4×10^7	50
No EDTAA	5×10^7	3	1.5×10^6	97
0.1 % EDTAA	7×10^7	0.7	5.4×10^7	23
No EDTAA	7×10^7	0.7	3.5×10^7	50

Phage 29. In one particular batch of broth phage 29 adsorbed slowly on to its own propagating strain. Also, very little adsorbed phage could be recovered as infective centres, thus giving results resembling phage 3A-infected cells + EDTAA. The addition of CaCl_2 to the cells before adding phage resulted in more efficient cell infection. Table 7 indicates that in this broth the phage had a threshold requirement of added CaCl_2 which was between 100 and 200 $\mu\text{g./ml.}$, since in unsupplemented broth and in broth + 100 $\mu\text{g. CaCl}_2/\text{ml.}$, only 18 and 19 % respectively of the adsorbed phage was recovered.

Table 7. *Infection of staphylococcus PS 29 with phage 29 in broth and in broth + CaCl_2*

Each tube contained phage + cells and the amount of CaCl_2 indicated.

Tube	Amount of CaCl_2 ($\mu\text{g./ml.}$)	Phage adsorbed	Phage in cells	Adsorbed phage lost (%)
1	—	61	11	82
2	100	68	13	81
3	200	66	46	30
4	300	68	39	43
5	400	65	40	38

Phage/0.01 ml.; mean of 4 replicates

The substitution for staphylococcus PS 29 of another strain R 5310, resulted in more rapid adsorption of phage, but the cation requirements were the same and even in 200 $\mu\text{g. CaCl}_2/\text{ml.}$ only 42 % of the adsorbed phage was recovered. Nevertheless, when infection was established in these suspensions of cells + CaCl_2 and EDTAA added 2 and 6 min. later, inactivation of adsorbed phage could be demonstrated (Table 8).

Table 8. *Effect of time of addition of EDTAA on recovery of adsorbed phage from cells of staphylococcus R 5310 infected by phage 29*

Each tube contained phage + staphylococci and except in Tube 1 CaCl_2 , 200 $\mu\text{g./ml.}$ EDTAA (to 0.1 %) was added at the times shown.

Tube	EDTAA added	Phage absorbed	Phage in cells	Adsorbed phage lost (%)
1	Nil	109	9	92
2	Nil	108	63	58
3	At 2 min.	106	22	79
4	At 6 min.	115	31	73
5	At 10 min.	124	54	61
6	At 20 min.	137	74	62

To summarize, shortly after adsorption, phages 3A and 29 pass through a period during which they can be inactivated by the addition of a chelating agent. This inactivation is rapid and irreversible and protects the cell from the lethal action of the phage. A reasonable hypothesis to explain these findings is that in the absence of divalent cations phage DNA cannot penetrate the cell. The divalent cations may therefore be essential to the process of penetration.

Effect of divalent cations in penetration

The experiment with a chelating agent in broth did not distinguish between a specific Ca ion effect and one due to divalent cations. The phenomenon of penetration was therefore examined by another method. When staphylococci are grown in citrated broth and resuspended in distilled water to which appropriate concentrations of cations have been added, the cocci adsorb phage which can be recovered as infective centres when the plating is done within 30 min. of infection. It can be argued that, under these conditions, phage adsorption had resulted in the first step only and that the later steps proceeded when the cells were transferred to the plating medium which contained adequate amounts of Ca ion. When, however, differences in the activity of various cations can be demonstrated under such conditions, then it may be assumed that penetration has taken place. Such differences were found. In experiments of this kind complete recovery of added phage was rare, partly because of inactivation of the free phage in distilled water and partly because of a proportion of abortive adsorptions. Nevertheless, the method can be used to compare the relative activity of equivalent amounts of cations.

Young cells grown in 0.5% (w/v) Na citrate broth were centrifuged and resuspended in distilled water. Samples (0.8 ml.) were distributed into tubes containing 0.1 ml. water or appropriate concentrations of CaCl_2 , MgCl_2 or SrCl_2 ; 0.1 ml. volumes of phage suspension (diluted in water) were then added. After 10 min. adsorption the tubes were centrifuged, the supernatant fluid, and the cells resuspended in water were plated.

Before penetration can occur the first step of adsorption must take place. This can be measured by the disappearance of free phage from the supernatant but is complicated by the fact that some phages are unstable in the salt solutions in absence of cells and it is not always possible to be certain whether their disappearance in the salt+cell suspensions is due to adsorption and inactivation or to inactivation while unadsorbed. In a previous paper (Rountree, 1951), basing evidence for lack of adsorption on the similarity between amounts of free phage in salt solutions and in salt solutions+cells, it was concluded that phages 7 and 42E had specific Ca ion requirements for adsorption. The present results, however, indicate that small amounts of phage adsorb and penetrate in the presence of Mg or Sr ion and small amounts of 42E in the presence of Mg but none in the presence of Sr ion.

The phages fell into four groups according to the relative activity of the three cations (Table 9). All the phages which showed penetration in SrCl_2 belonged to group A. On the other hand, the group B phages showed no Sr

activity and in three cases, comparatively little Mg activity. This lack of Mg or Sr activity was not due to failure of adsorption, since the free phages were relatively stable in these salt solutions in the absence of cells. Phage 42 E, which is group A serologically, was aberrant in its behaviour.

Table 9. *Relative activity of Ca, Mg and Sr in penetration of staphylococcal phages*

++ = cations equally active; ± = cations partially active compared with Ca; - = no activity.

Ca	Mg	Sr	Phages
Relative activity			
++	++	++	3B, 6, 7, 47
++	++	±	3A, 3C, 47A, 51
++	++	-	29, 29A, 31A, 31B, 42D, 52A
++	±	-	31, 42E, 44, 52

The results indicate that, save with phages 31, 42E, 44 and 52, no specific Ca ion effect was demonstrated for phage penetration and that even in the exceptions there was partial activity with Mg ion. The stimulatory effect of Ca ion must therefore occur at a later stage in phage growth.

The effect of calcium towards the end of the latent period

Since the addition of CaCl_2 to broth increased the average burst size with all the phages examined and in some cases converted an unsuitable broth to one which gave a good yield of phage, a chelating agent added to the infected cells after penetration has occurred should decrease the average burst size. Na citrate was found to have this effect, but owing to its inactivation of free phage, doubt existed as to the point in the growth cycle at which it acted. The effect of 0.1% EDTAA on the average burst size and growth curves of phages 3A and 29 was therefore investigated. This concentration of EDTAA (0.1%) did not completely inhibit phage production but did cause a marked decrease in average burst size. Figs. 3 and 4 show that this depressive effect occurred whether the chelating agent was added relatively early or late in the latent period. Phage 3A showed a similar degree of decrease in phage yield/cell, whether the EDTAA was added at 20, 30, 40 or 50 min. after infection. Similarly, phage 29 gave lower average burst sizes when EDTAA was added at 10 or 50 min. In the latter case, phage release was already occurring when the chelating agent was added. This depressive action of EDTAA could be partially reversed by the addition of CaCl_2 to the infected cells, provided this was done before phage release was completed (Table 10). It was concluded that in these phages the specific Ca ion requirements were concerned with a very late step in phage synthesis.

The failure of phages of group B, e.g. 42C, 42D, 44 and 52, to produce phage in the basal broth or broth + MgCl_2 was not due solely to failure of penetration. When Mg ions were adequate for penetration, successive platings of the infected cells showed their gradual failure to register as infective centres. It

would seem therefore that, in the absence of adequate amounts of Ca ion these phage + cell complexes gradually lost their ability to complete a step in phage multiplication. The present data do not, however, allow a distinction between an early and late need for Ca ion in these phages.

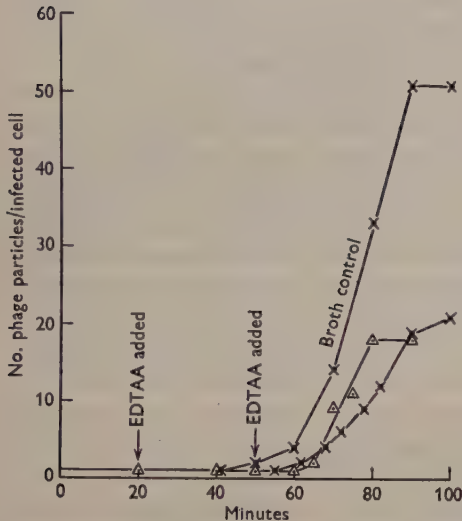


Fig. 3.

Fig. 3. The effect of EDTAA (0.1 %, w/v) on the one-step growth curve in broth of phage 3A. \triangle — \triangle = EDTAA added at 20 min.; \times — \times — \times = EDTAA added at 50 min.

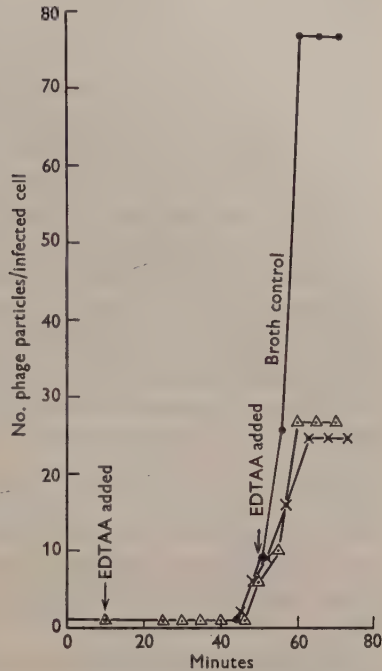


Fig. 4.

Fig. 4. The effect of EDTAA (0.1 %, w/v) on the growth curve in broth of phage 29. \triangle — \triangle = EDTAA added at 10 min.; \times — \times — \times = EDTAA added at 50 min.

Table 10. *Partial annulment by CaCl_2 of the effect of EDTAA on the average burst size of phage 3A*

Tube no.	Contents of tube	Average burst size
1	Phage and cells in broth	42
2	Phage and cells in broth + 100 $\mu\text{g./ml.}$ CaCl_2 at 1 min.	70
3	Phage and cells in broth + 0.1 % EDTAA at 15 min.	10
4	Phage and cells in broth + 0.1 % EDTAA at 15 min. + CaCl_2 at 50 min.	40
5	Phage and cells in broth + 0.1 % EDTAA at 15 min. + CaCl_2 at 60 min.	20
6	Phage and cells in broth + 0.1 % EDTAA at 15 min. + CaCl_2 at 70 min.	22

DISCUSSION

From the present results and those previously reported, a general outline of the role of divalent cations in the growth of staphylococcal typing phages may be suggested. It should be understood, however, that the quantitative

details vary from phage to phage. Adsorption of the phages to their host cells requires divalent cations and can be inhibited by suitable chelating agents. Following adsorption, the phage particle passes through a short period during which it can be irreversibly inactivated by the removal of divalent cations from the medium. Although there is no direct evidence, it seems reasonable to assume that this inactivation is due to the abortive separation of phage protein and deoxyribonucleic acid at the cell surface and the inability of the deoxyribonucleic acid to penetrate the cell in the absence of divalent cations. With the possible exception of phage 42E, there is no evidence of any specific Ca ion requirements in these steps. There is, however, evidence of a correlation between antigenic structure and cation requirement since the group B phages are unable to use Sr ion for penetration.

After this 'vulnerable' period has passed, the phage material is no longer inactivated by the chelating agent EDTAA and infected cells treated with this substance from 10 min. onward in the latent period will register as infective centres when plated. There is, however, a specific calcium requirement late in the latent period. This is shown by the increase in average phage yield/cell in increasing concentrations of Ca ion and in the depression in yield in the presence of a chelating agent. Particularly with the group A phages, the amount of CaCl_2 which stimulates phage yield is much greater than that required for adsorption or penetration; for example, phage 3A requires $10\text{ }\mu\text{g}$. CaCl_2/ml . for adsorption and $100\text{ }\mu\text{g}$. CaCl_2/ml . for maximal stimulation. In a few cases, some stimulation of yield can be obtained with Mg or Sr but neither cation is as effective as an equivalent amount of Ca and generally no activity is found.

The results do not exclude the possibility that Ca ion is also required specifically for some process in phage synthesis which immediately follows penetration, as suggested for other phages by Fildes (1953); but they do indicate the importance of Ca ion in the final stages of phage multiplication. It is unlikely that the effect of EDTAA is a direct one on the metabolism of the host cells since in some experiments in which a decrease in phage yield was obtained the chelating agent was in contact with the cells for only 5–10 min. before they were plated. There is a possibility that the depressive effect of the chelating agent was due to its direct inactivation of completed phage particles not yet liberated from the cell. Since, however, free phage particles after liberation were not affected by the concentration of chelating agent used, this seems unlikely. It is possible that the effect of the chelating agent is an indirect one in making Ca unavailable to not yet completed phage particles or to some enzyme or enzymes concerned in phage synthesis. The stabilizing effect of Ca ions on various proteins and enzymes is a well-recognized biological phenomenon and their activity in the synthesis of the staphylococcal phages may be of a similar nature.

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The Induced (Adaptive) Biosynthesis of β -Galactosidase in *Staphylococcus aureus*

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SUMMARY: *Staphylococcus aureus* strain Duncan readily synthesized the adaptive (inducible) enzyme β -galactosidase in washed suspension when supplied with a mixture of amino acids and an inducer. The amount of enzyme produced was a function of the quantity of amino acids supplied and the rate of synthesis was determined by the concentration of inducer in the system. Galactose was a highly active inducer, lactose a relatively feeble one. Production of the enzyme ensued after a lag period which could be shortened by the addition of glucose or sodium lactate and further shortened by supplying a mixture of purines and pyrimidines to the system. Enzyme formation was strongly inhibited by various antibacterial agents.

It is generally accepted that enzymic adaptation in micro-organisms is a consequence of the synthesis of an enzymically active protein under the influence of an inducer which is usually the substrate or a reaction product of the enzyme in question. Monod, Pappenheimer & Cohn (1952) showed that the adaptive β -galactosidase of *Escherichia coli* appears to be inducible exclusively under conditions which allow the synthesis of new protein. Similarly, Halvorson & Spiegelman (1952, 1953*a, b*) concluded that the synthesis of maltozymase in *Saccharomyces cerevisiae* strain K was from free amino acids. They also showed that enzyme formation was inhibited by a number of amino acid analogues. Gale & Folkes (1953*a, b*) found that an increase in the protein content of cells of *Staphylococcus aureus* occurred when these were incubated with glucose and a mixture of those amino acids which were essential nutrients for this organism, protein synthesis being stimulated by the addition of a mixture of purines and pyrimidines. Protein synthesis was inhibited by bactericidal concentrations of aureomycin, terramycin and chloramphenicol and by higher concentrations of other antibiotics. The work to be described was undertaken to ascertain whether the conditions necessary for protein synthesis as described by these authors also applied to the synthesis of adaptive enzymes in *S. aureus*. Price (1952) found that *S. muscae* would grow on lactose after a short lag period: in the present work it is shown that the enzyme β -galactosidase is adaptive in *S. aureus*. A preliminary note by Gale & Folkes (1954) has described the development of β -galactosidase activity in broken *S. aureus* preparations where enzyme formation required a source of energy, a complete mixture of amino acids and an inducer.

METHODS

Organism and growth medium. The Duncan strain of *Staphylococcus aureus* used by Gale & Folkes (1953*a, b*) was also used in this work. The growth medium was that of Gale (1951); it contained salts, 0.1 % (w/v) Marmite, 0.2 % (w/v) arginine and 0.1 % (w/v) glucose and was prepared in batches of 2 l. in 5 l. flasks. The organisms were harvested after 15 hr. static growth at 37°, washed once in distilled water and resuspended in distilled water. The dry weight of suspensions was estimated by means of a Hilger 'Spekker' absorptiometer previously calibrated for this organism.

Growth inhibition tests. The presence or absence of growth was recorded after 15 hr. incubation at 37° of an inoculum of *c.* 10^7 cells in 5 ml. growth medium containing the growth inhibitory agent under test.

Estimation of β -galactosidase. The method was based on that of Lederberg (1950) using as substrate *o*-nitrophenyl- β -D-galactopyranoside (ONPG) synthesized by the method of Seidman & Link (1950). It was found with some cell suspensions that hydrolysis of ONPG was enhanced by adding cysteine to the test system. The following procedure was therefore adopted. A sample of the culture under test was centrifuged at 3000 g., the cells washed in 3.0 ml. 0.2 M-sodium phosphate buffer (pH 7.5) and resuspended in 3.0 ml. of this buffer. Cysteine (20 mg./ml.; 1.0 ml.) was added and, after temperature equilibration in a 37° water bath, 1.0 ml. of a solution of ONPG (0.033 M). The incubation was continued for a further 30 min. and the cells removed on the centrifuge. The concentration of *o*-nitrophenol in the supernatant was estimated by measuring the absorption at 420 m μ . using a Beckman spectrophotometer. The readings so obtained were converted to 'enzyme units', one unit being defined as the amount of enzyme liberating 1 m μ mole *o*-nitrophenol/hr. Under these conditions a linear relationship was obtained between absorption and enzyme activity over the range 0 to 10,000 units.

*Induction of β -galactosidase activity in washed suspensions of *Staphylococcus aureus*.* The following inducing system was adopted as standard (quantities/ml.): 400 μ g. each of: L-isomer of DL-alanine, L-aspartic acid, L-cysteine, glycine, L-glutamic acid, L-histidine, L-arginine, L-leucine, L-valine, L-tyrosine, L-proline, L-lysine, DL-serine, DL-isoleucine, DL-threonine, DL-methionine, DL-phenylalanine, DL-tryptophane; 1 μ g. each of adenine, guanine, hypoxanthine, thymine, uracil and xanthine; 1 mg. of D-glucose; 20 mg. of D-galactose; 2.0 mg. dry weight washed suspension of *S. aureus*; 0.0086 M-KH₂PO₄; 0.026 M-NaCl; 0.0042 M-MgSO₄.7H₂O; and 0.035 M-Na₂HPO₄. The system was maintained at 37° in a water-bath and a stream of air bubbled through it.

RESULTS

Some properties of Staphylococcus aureus β -galactosidase

Effect of growth conditions. Cells grown in the normal medium had a β -galactosidase activity of 10–20 units/mg. dry wt. cells. When the glucose in the medium was replaced by 2 % (w/v) lactose or 2 % (w/v) galactose the

activities of the resulting cells were *c.* 2000 and 4000 units/mg. dry wt. respectively. Similar differences were observed in the ability of the cells to ferment or oxidise galactose or lactose. The activity of cells grown on glucose was of the same order as that of cells grown without added sugar, showing that the lack of enzyme was not due to suppression by glucose and that the differences observed were of an adaptive nature. Such adaptive differences

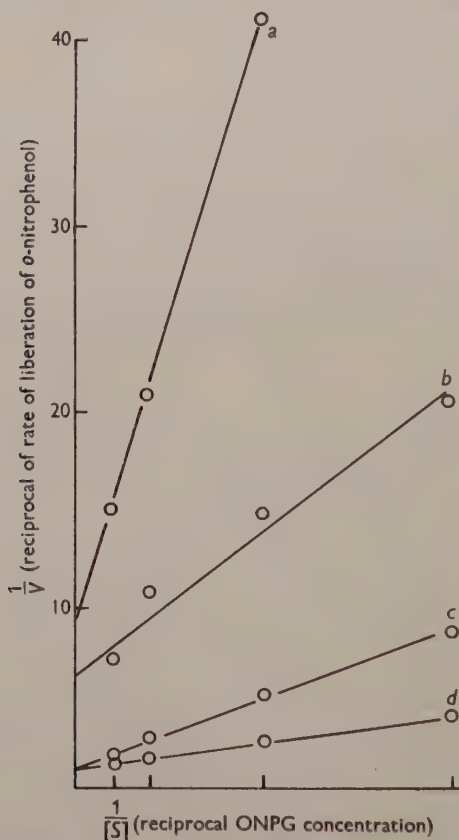


Fig. 1. Effect of lactose, glucose and galactose on ONPG hydrolysis. 1.0 mg. dry wt. cells suspended in 3.0 ml. 0.2 M-sodium phosphate buffer (pH 7.5), 1.0 ml. cysteine solution (20 mg./ml.) and 1.0 ml. ONPG solution (0.033 M). Also added; (a) lactose (final concentration 100 μ g./ml.); (b) glucose (final concentration 10 mg./ml.); (c) galactose (final concentration 10 mg./ml.); (d) no additions. Data plotted according to the method of Lineweaver & Burke (1934).

were not observed with maltozymase, sucrozymase, melibiozymase, raffinozymase, and enzyme systems which oxidized sodium lactate or sodium acetate. This organism showed no urease, tetrathionase or ability to oxidize sodium benzoate.

Effect of pH. β -Galactosidase activity of galactose-grown cells was constant over the pH range 6.0–8.0.

Effect of substrate concentration. The β -galactosidase in galactose-grown cells had a Michaelis constant of 4×10^{-4} M for ONPG hydrolysis and was competitively inhibited by galactose and non-competitively by lactose and glucose (Fig. 1). The K_i for lactose inhibition was 0.37×10^{-5} M, showing that this *Staphylococcus aureus* β -galactosidase has a higher affinity for lactose than for the synthetic β -galactoside. This is the opposite of the situation found with neurospora lactase (Landman & Bonner, 1952), *Escherichia coli* β -galactosidase (Lederberg, 1950; Cohn & Monod, 1951) and almond emulsin (Helferich, 1951; Pigman, 1944).

Attempts to obtain a cell-free β -galactosidase preparation. An active extract was not obtained by treatment with acetone, toluene, butanol or detergents such as cetyltrimethylammonium bromide, Aerosol OT and Tween 80; by lyophilization; by disintegration on the vibrator of Mickle (1948) or in the press of Hughes (1951) or by exposure to ultrasonic vibration; by grinding with alumina (McIlwain, 1948).

Factors affecting β -galactosidase synthesis in washed suspensions of Staphylococcus aureus

Cells grown in the glucose-containing medium described above and then incubated in the standard induction system showed a large increase in their ability to hydrolyse ONPG and to oxidize or ferment lactose or galactose. These activities increased together but for most of the experiments only the ability to hydrolyse ONPG was tested. Under the standard conditions the β -galactosidase activity remained constant at the 'unadapted' level of *c.* 20 units/mg. dry wt. over the first hour of incubation (Fig. 2), then increased almost linearly at a maximum rate of *c.* 2000 units/mg. dry wt./hr. (Fig. 3) up to a maximum content of *c.* 4000 units/mg. dry wt. after about 3–4 hr. incubation (Fig. 7). The activity then decreased; the rate of decline was very variable from one experiment to another (cf. Figs. 6, 7).

Nature and concentration of inducer. Monod, Cohen-Bazire & Cohn (1951) showed that β -galactosidase formation in *Escherichia coli* was induced by lactose, melibiose and galactose, the first two being more efficient than galactose. Galactose is the most efficient inducer in the *Staphylococcus aureus* system (Fig. 2). Lactose was only about 1 % as active as galactose, whilst melibiose, raffinose, arabinose and galacturonic acid were inactive. The rate of adaptation was proportional to the concentration of galactose present (Fig. 3).

Effect of amino acids. The maximum amount of adaptive enzyme produced was dependent on the concentration of amino acids in the suspension (Fig. 4), the length of the lag phase and the rate of formation being independent of the amino acid concentration. Omission of tryptophan or phenylalanine from the system abolished enzyme formation while omission of cysteine, which was found by Gale & Folkes (1953*a*) to be essential for protein synthesis in this organism, decreased the amount of enzyme produced by 62 %.

Effect of 4-fluorophenylalanine. When enzyme formation was limited by the amount of phenylalanine in the test system there was a competitive inhibition

of phenylalanine utilization by 4-fluorophenylalanine. The effect of the addition of phenylalanine was maximal at a concentration of 25 mg./ml. When the data were plotted according to the method of Lineweaver & Burke (1934) the curve obtained consisted of two linear portions (Fig. 5). This type of curve is stated to be due to limitation of the reaction by diffusion of the substrate

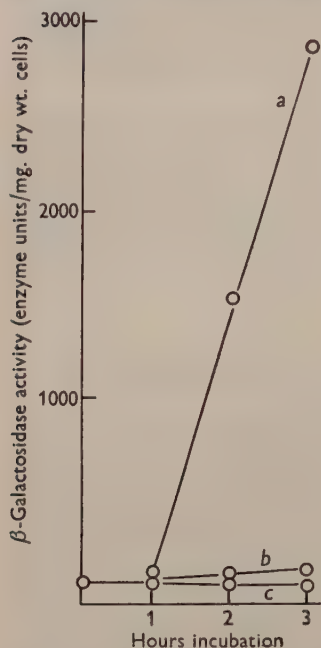


Fig. 2.

Fig. 2. Effect of various inducers on β -galactosidase formation. Basal system contained amino acids, glucose, salts, purines and pyrimidines. Inducer added to a final concentration of 20 mg./ml. as follows: (a) galactose, (b) lactose, (c) no inducer, melibiose, raffinose, arabinose or galacturonic acid.

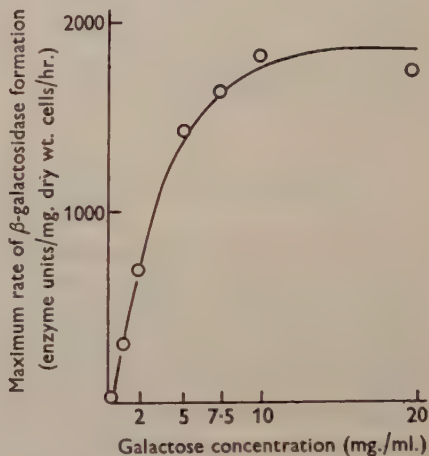


Fig. 3.

Fig. 3. Effect of galactose concentration of β -galactosidase formation. Standard conditions with galactose concentration as shown.

(phenylalanine in this case) and is common in reactions which occur in intact cells. No effect of 5-fluorotryptophan in concentrations up to 400 times that of the tryptophan present could be detected.

Effect of glucose, purines and pyrimidines. The synthesis of β -galactosidase is paralleled by the production of lactozymase and galactozymase. Consequently, when lactose or galactose is used as an inducer enzymic adaptation leads to the utilization of the inducer as a source of energy. In order to diminish the dependence of the induction upon energy supplied by the inducer, a source of energy which would be immediately available without adaptation was added as well as the inducer. Addition of glucose or sodium lactate stimulated the adaptation, principally by decreasing the period of lag; the rate of formation was unaffected but a higher maximum activity was

produced (Fig. 6). The optimal concentration of glucose was 1 mg./ml. (higher concentrations were inhibitory); lactate had an optimal effect at 10 mg./ml. In order to eliminate possible changes in the cells due to the change in carbon source, glucose was used as an energy source for growth and for adaptation

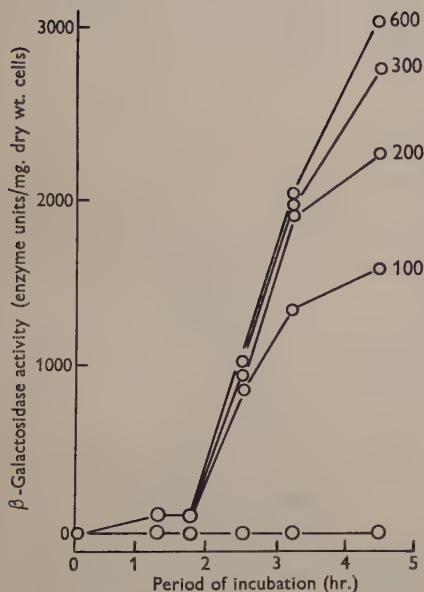


Fig. 4.

Fig. 4. Effect of amino acids on β -galactosidase formation. Standard conditions with the concentration of amino acids as shown (μ g. of each amino acid/ml.).

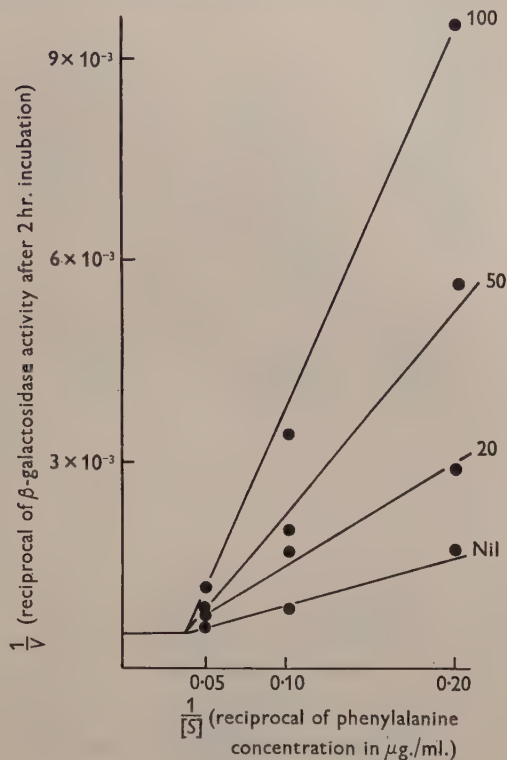


Fig. 5.

Fig. 5. Effect of 4-fluorophenylalanine on β -galactosidase formation. Conditions standard except for the concentration of DL-phenylalanine in the system and the addition of 4-fluorophenylalanine to the final concentration (μ g./ml.) shown. Data plotted according to the method of Lineweaver & Burke (1934).

procedures. Purines and pyrimidines also diminished the lag period (Fig. 7). In a system containing galactose, amino acids and salts the effect of purines and pyrimidines was about doubled by the presence of glucose; the stimulation of enzyme formation by glucose itself was 20 % greater in the presence of purines and pyrimidines (Table 1).

Inhibition of enzyme formation

Table 2 shows the effects of various compounds on adaptive enzyme formation. The substances can be divided into three groups: (i) the most active, which completely inhibited adaptation at the same concentration as that

required to inhibit growth, were neomycin, 2:4-dinitrophenol, puromycin, tetracycline, aureomycin, terramycin and chloramphenicol; (ii) considerable but not completely inhibitory activity at the growth-inhibiting concentration was shown by usnic acid, bacitracin, dihydrostreptomycin and sodium azide;

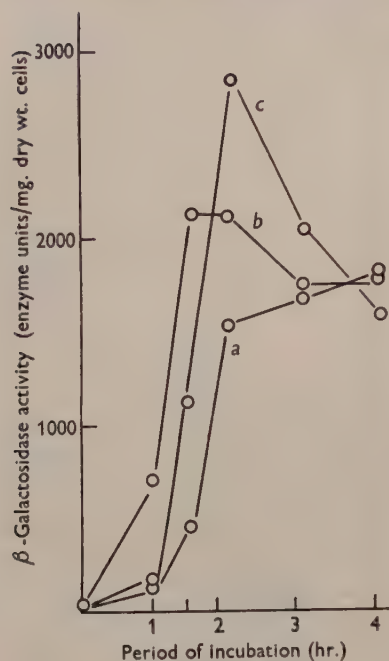


Fig. 6.

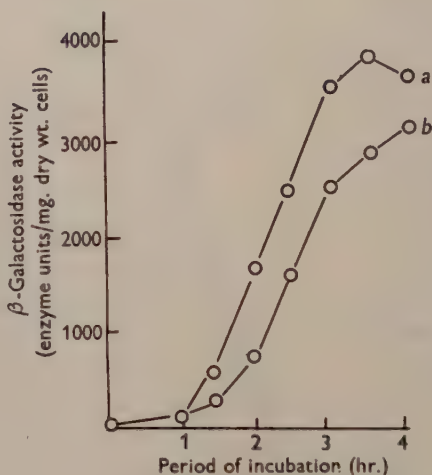


Fig. 7.

Fig. 6. Effect of energy source on β -galactosidase formation. Standard conditions except for the energy source which was: (a) nil; (b) sodium lactate (10 mg./ml.); (c) glucose (1 mg./ml.).

Fig. 7. Effect of purines and pyrimidines on β -galactosidase formation. Curve (a) standard conditions; curve (b) purines and pyrimidines omitted.

Table 1. *Effect of glucose, purines and pyrimidines on β -galactosidase formation*

Cells were grown in glucose-containing medium and made into a washed suspension before incubation for 2 hr. at 37° in a standard system containing salts, amino acids and galactose. β -Galactosidase activity was determined after 2 hr. incubation and expressed as enzyme units/mg. dry wt. cells.

Further additions to system	β -galactosidase activity	Increased activity due to	
		Glucose	Purines and pyrimidines
(a) Nil	368	—	—
(b) Glucose (1 mg./ml.)	1184	816	—
(c) Purines and pyrimidines (1 μ g./ml. of each)	504	—	136
(d) Glucose + purines + pyrimidines concentrations as (b) and (c)	1488	984	304

(iii) penicillin and streptomycin were active only at concentrations considerably higher than those required to inhibit growth (penicillin never completely prevented adaptation); actidione and gramicidin did not prevent growth at high concentrations but had some inhibitory effect on β -galactosidase formation.

Table 2. *Inhibition of β -galactosidase formation*

Washed cells harvested from glucose-containing medium incubated at 37° in the standard system. β -Galactosidase activity was determined after 2 hr. incubation and expressed as percentage inhibition of formation in the presence of the inhibitor as compared with a control without inhibitor. The growth inhibitory concentration is the minimum concentration of inhibitor which will completely prevent growth in a fully nutrient medium.

Compound	Growth inhibitory concentration ($\mu\text{g./ml.}$)	Inhibition of β -galactosidase formation	
		Concentration of inhibitor ($\mu\text{g./ml.}$)	Inhibition of β -galactosidase formation (%)
Aureomycin	0.03	0.03	97
Terramycin	0.03	0.03	97
Tetracycline	0.10	0.10	93
Chloromycetin	10	10	98
Neomycin	3	3	93
Puromycin	10	10	100
2:4-Dinitrophenol	100	100	97
Usnic acid	30	30	67
	—	100	100
Bacitracin	30	30	67
	—	100	100
Dihydrostreptomycin	30	30	56
	—	100	95
Sodium azide	100	100	45
	—	300	77
	—	1000	100
Streptomycin	30	30	6
	—	100	70
	—	300	94
Penicillin	0.01	0.01	5
	—	0.10	30
	—	1.00	73
	—	1000	73
Actidione	*	3000	72
Gramicidin	*	3000	25
Polymyxin	3000†	3000†	100

* = no growth inhibition at 3000 $\mu\text{g./ml.}$;

† = cells lysed by polymyxin.

DISCUSSION

The general similarity between the results presented above and those obtained by Gale & Folkes (1953*a, b*) leaves little doubt that enzymic adaptation in *Staphylococcus aureus* is controlled by the same factors as those which affect protein synthesis. This conclusion is supported by the fact that there is no adaptation in the absence of amino acids and that the effect of amino acids

can be abolished by the presence of an inhibitory analogue of one of them; it is in line with the conclusions of Monod *et al.* (1952) and of Halvorson & Spiegelman (1953*a, b*). The fact that glucose and a supply of purines and pyrimidines shorten the lag period indicates that these compounds accelerate some reaction which occurs during this lag and which must take place before formation of adaptive enzyme can begin. It is probable that this reaction is the synthesis of nucleic acid and that this synthesis must occur before adaptive enzyme formation can take place, as suggested by Gale & Folkes (1954).

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Factors Involved in the Production of a Novel Kind of Derangement of Storage Mechanism in Living Holotrich Ciliate Protozoa from Sheep Rumen

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SUMMARY: When metabolizing glucose or other fermentable sugar *in vitro* at a temperature 8-12° below the normal rumen temperature, three species of rumen holotrich ciliates were liable to exhibit highly abnormal appearances which were not seen at 35-38°. These abnormal appearances consisted essentially in a contraction and fusing together of the bulk of the storage polysaccharide (amylopectin) granules into a narrow central zone in the endoplasm. The outer clearer endoplasmic region, however, still contained numerous discrete granules in *Isotricha prostoma* and *I. intestinalis* but not in *Dasytricha ruminantium*. The abnormality was not quickly lethal to the organisms and appeared not to involve any alteration in shape or position of the macro-nucleus. The presence of rumen liquor, with all its soluble constituents but not necessarily its bacteria, is required for a high incidence of abnormality and the organisms should also initially contain but little storage polysaccharide. Evidence is presented in favour of the view that the abnormalities are connected with the utilization (auto-fermentation) of storage polysaccharide rather than with its synthesis. Nevertheless, the abnormalities cannot be induced in *Isotricha* when starch grains (vegetable or protozoan) have first been ingested.

When harvesting in quantity from sheep rumen contents the holotrich ciliates (i.e. a mixture of *Isotricha prostoma*, *I. intestinalis* and *Dasytricha ruminantium*) by a labour-saving and apparently quite reproducible modification of the glucose-fermentation methods of Masson & Oxford (1951) and Heald & Oxford (1953), we noted in our washed suspensions what seemed to be the sporadic and unpredictable appearance of a relatively few highly abnormal but still living ciliates. Furthermore, the kind of abnormal appearance in the *Isotricha* organisms was not exactly the same as in the *Dasytricha* organisms, although a marked contraction of internal contents was a feature in each. This paper records our observations on: (a) how to prevent these abnormalities from occurring; (b) how to ensure their appearance in a large percentage of living ciliates in the culture; (c) the nature of the functional derangement which can bring about such abnormal appearances in still living organisms.

METHODS

Rumen contents of sheep on various diets as source of holotrich ciliate protozoa. For a reason to be stated later, it was important to know whether the experimental animal was being fed an appreciable amount of vegetable starch and if so whether this consisted in large part of grains small enough to be swallowed by the *Isotricha* spp. (cf. Sugden & Oxford, 1952). Table 1 lists the rumen-

fistulated sheep used at various times in this study, together with relevant details concerning their respective rations. By microscopic examination of the concentrates fed (for composition see Table 1) it was established that a high proportion of the constituent starch grains were in fact small enough to be swallowed by the *Isotricha* spp. (This would not have been so had potato starch been fed.) The ration of sheep no. 1004 was changed during the experiment because the population of holotrichs in its rumen had dwindled almost to zero. Supplementation of hay by fodder beet, which contains relatively much soluble sugar and little starch, did in fact soon cause the holotrich population to increase. Unfortunately a similar dwindling of the holotrich population also took place in another sheep (no. 272) maintained on a starch-free diet, but on account of its age it was not thought advisable to change the ration in this instance.

Table 1. *List of the rumen-fistulated sheep used and their rations*

Ref. no. of sheep	Age (years)	Ration (g./day)	Vegetable starch in ration
272, Suffolk cross	7	Hay: 1200 Yeast: 40	0
879, Cheviot	4	Hay: 750 Concentrates:* 450	+ + + (small grains)
997, Grey face	4	Hay: 500 Concentrates: 450	+ + + (small grains)
1004, Cheviot	5	Hay only at first Later: Hay: 1500 Fodder beet: 800	0 +, but grains mostly too large to be swallowed by <i>Isotricha</i> spp.
8060, Cheviot	3	Hay: 800 Grass cubes: 400	Almost 0

* Concentrates: 4 parts maize; 1 part oats; 1 part bran; $\frac{1}{2}$ part linseed; $\frac{1}{2}$ part white fish meal.

Method of separation of holotrich ciliates from strained rumen contents. The soluble carbohydrate fermentation leading to deposition of holotrichs as a bottom layer after polysaccharide storage had taken place was carried out as described by Heald & Oxford (1953), the strained rumen liquor being allowed to stand for 1 hr. at 38° before the removal of scum. The removal of scum and the subsequent transfer to funnels of 250 ml. capacity was carried out at about 15° (i.e. room temperature). Glucose or other sugar was added to give a final concentration of usually not more than 0.75% (w/v), after which the funnels were placed in a 38° incubator. It was later found that the temperature of the rumen liquor at this point was seldom above 33°. When it was realized that this method sometimes led to the deposition of abnormal organisms, many modifications in procedure were made, some of which are described later under 'Results'. Temperature was one factor which was brought more closely under control, and when it was desired quickly to cool or warm the contents of a separating funnel to a desired temperature, a current of cold or hot water from an Ascot heater was directed on to the wide part of the funnel while the

liquid within was kept swirling by rotation of the funnel by hand. At suitable intervals of time the funnel was brought to rest and the temperature of its contents taken. The procedure was repeated until the desired temperature was reached.

Preparation of washed and starved suspensions of living holotrichs for in vitro experiments. The white bottom layer which settled during glucose fermentation was withdrawn after various periods of time into boiling- or test-tubes nearly filled with phosphate-acetate buffer of the following composition (% , w/v): NaCl, 0.5; anhydrous sodium acetate, 0.13; KH_2PO_4 , 0.03; K_2HPO_4 , 0.10; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; and sometimes $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 0.01. Any necessary adjustment of pH required to reach the value 7.2 (6.5 when the buffer contained Ca) was made with dilute caustic soda or acetic acid. After washing by decantation the protozoan suspensions were kept overnight at 38° and examined next morning for living and relatively empty organisms. Sometimes the starvation period had to be extended for a day, with one or more changes of buffer, in order to obtain organisms sufficiently depleted of storage polysaccharide to give a visible response to added glucose. When the protozoan suspensions contained much debris and dead protozoa these could mostly be removed by allowing the motile organisms to swim through a layer of fine-meshed St Martin's 25N nylon bolting cloth attached to the mouth of a boiling tube filled with buffer. The above stable buffer, which contains no bicarbonate, is the simplest possible for maintenance of the holotrichs, although of course it bears no close resemblance to the salt content of rumen liquor, save perhaps with respect to total Na + K content.

Preparation of 'partly' and 'totally' cleared rumen liquor for use as suspending media. It was important to find whether rumen bacteria or their products were responsible for the observed abnormalities. To obtain rumen liquor free from ciliate protozoa but still containing most of its bacteria, i.e. 'partly' cleared, the top layer of strained rumen liquor from which scum had been removed was centrifuged 2 or 3 times for 3 min. at 2200 g and the highly turbid supernatant fluid used. Totally cleared rumen liquor (i.e. free from both protozoa and bacteria) was this supernatant fluid from which all or nearly all particulate matter had been removed by centrifuging twice at 11,000 g for 20 min.

Examination of holotrich suspensions for abnormal organisms

(a) *Direct microscopy of living ciliates in wet preparations.* When the organisms were moving too rapidly, a brief cooling of the slide preparation in the refrigerator soon slowed them down sufficiently for observation with the high dry objective. It is fortunately not at all easy, even by prolonged cooling, to stop all ciliary motion in these anaerobic ciliates. The living ones always regain motility on warming up again.

(b) *Staining with Lugol's iodine.* The holotrichs, even when relatively empty, still stain solidly brownish purple with excess of iodine because of the highly iodophilic granules of storage polysaccharide which still remain. In order to ensure that a few organisms at least would take the stain only in part it was

best to add a very little Lugol's iodine to a living suspension on a slide and then to add a further small drop of the same suspension of organisms, mixing well.

(c) *Rapid staining with acetocarmine for protozoan macronuclei.* When the organisms are filled with storage starch it is difficult to determine the exact position of the oral region and in this condition overall shape is of little diagnostic value. The shape and situation of the macronuclei are however characteristic. When a drop or two of really saturated acetocarmine (Schneider; see Gurr, 1953, p. 296) is added to a drop of living suspension on a slide, the organisms are immediately killed and fixed by the acetic acid in the stain and their macronuclei become stained discernibly pink, after various periods up to 1 hr. at room temperature. The time required is proportional to the density of the storage polysaccharide. It sometimes helped to starve the suspension overnight before using the stain.

Permanent preparations

The difficulty here was to devise a method of fixing the ciliates to a coverslip which, without distorting the organisms, ensured that they remained attached throughout the subsequent changes of solution. This was best achieved by drying as much as possible in air without killing the ciliates in the drop and fixing rapidly by the flotation method on Bouin or Schaudinn fluids. The stains used were iron-haematoxylin or picro-carmine, whereby the large macronucleus was made clearly visible. *Isotricha prostoma* has a slightly curved and elongated macronucleus not centrally situated while *I. intestinalis* has a more egg-shaped macronucleus in the centre of the organism. The macronucleus of *Dasytricha ruminantium* is more or less spherical and central in position (see Kudo 1950, p. 606; but his fig. 287 c is clearly *I. intestinalis* and fig. 287 d *I. prostoma*, not vice versa as stated).

The micronucleus is only seen with difficulty in Feulgen preparations and appeared to be of no diagnostic value in this investigation. The use of horse serum or glycerol albumin for fixing the ciliates to the coverslip did not give successful results nor did the use of eosin or light green as counterstains greatly improve the preparations.

RESULTS

Nature of the abnormalities in the three species of holotrichs

As previously shown (Oxford, 1951) the metabolism of glucose by these ciliates very rapidly leads to a great increase in the numbers of storage polysaccharide (amylopectin) granules of quite uniform size resembling yeast cells. These granules are evenly distributed throughout the endoplasm (see Oxford & Sugden, 1953, for a photomicrograph of a suspension of these granules). Pl. 1, fig. 1, of the present paper shows five completely filled normal organisms, the smallest of which is *Dasytricha ruminantium* (this figure also shows one quite abnormal representative of this latter organism). When such normal

organisms are ruptured, the discrete grains stream out; they never seem to be stuck together in clumps. The abnormality here discussed, which was at times shown by holotrichs from all five sheep, is a markedly uneven distribution of storage granules in living and actively motile organisms. By direct microscopy of living ciliates, the granules are seen to be mostly condensed together into the inner region of the endoplasm as shown in Pl. 1, fig. 2, which exhibits all stages of 'compression' of the storage granules save the most extreme. In the middle stage of the process it might almost seem that a membrane had developed to enclose this differentiated inner zone (Pl. 1, fig. 3), but for the following reasons this is held to be unlikely. (1) Careful examination by both ordinary and phase microscopy with the high dry objective did not reveal a true membrane but only a slightly irregular boundary. (2) At a later stage in the life of the abnormal *Isotricha* organisms, a jagged fracture of the inner zone may occur (Pl. 1, fig. 4). It seemed as if the inner zone itself was rigidly solid, rather than a collection of a large number of discrete grains enclosed by a membrane. (3) This conclusion was confirmed by rupturing the organisms mechanically and then staining with iodine. The rupture could be achieved very simply by forcing the coverslip over the slide, by use of the thumb, with a simultaneous squeezing and shearing motion. When this operation was carried out with a drop containing only normal organisms an even distribution of discrete grains was obtained on rupture. A suspension which contained abnormal organisms, however, yielded numerous iodophilic 'lumps' of various sizes and shapes as well as discrete grains. Further examination of these lumps showed that they did not have a smooth margin, but parts of individual granules could be seen projecting. It could be clearly ascertained that the smallest lumps did in fact consist of only a few granules stuck together.

It will be seen from Pl. 1, fig. 2, 4 and 5 particularly, that the highly abnormal *Isotricha* organisms still contained some storage granules in the outer region of the endoplasm, whereas the *Dasytricha* organisms seemed to have none or very few indeed in this region. This conclusion was confirmed by overstaining with Lugol's iodine when nearly all the abnormal *Isotricha* organisms stained solidly, while the clear outer zone in abnormal *Dasytricha* organisms remained uncoloured or at most showed a faint diffuse reddish brown. When, however, the preparation was incompletely stained with iodine, as described under 'Methods', the outer zone in the endoplasm of the *Isotricha* organisms could be discerned in certain instances, and appeared to contain a soluble starch-like material as well as storage starch in granular form, since a distinct brownish purple hue, more or less evenly diffused, was perceptible in that region. The rapid acetocarmine stain also disclosed a clear outer region in the *Dasytricha* organisms, but the endoplasmic picture with this stain was complicated by the swelling of the organism as a whole, and particularly of the ectoplasm, because of the acetic acid.

A very clear indication of abnormality was shown in the permanent, fixed and nuclear stained preparations. The normal holotrich organism had an entire outline, while the abnormal organism had an extremely battered or crenated outline, as if the outer endoplasmic region had not withstood the

drying and fixing process, although both types of organism were on the same slide. The macronucleus showed no abnormality.

The nuclear stains revealed also that both species of *Isotricha* showed the characteristic abnormality, identical in each organism in the later stages, but perhaps not quite beginning in the same way in the two species. Thus, the early stage of 'compression' of the storage grains seemed to encompass the whole mass in *I. intestinalis* but sometimes occurred at one end only in *I. prostoma*.

Prevention of the abnormality

To avoid the possibly harmful fall in temperature when the scum was removed and the liquor was transferred to funnels (mentioned on p. 299), the procedure was modified so that the straining through gauze was carried out in the incubator. The filtrate, after removal of scum, was warmed to 38° by placing the beaker containing it in a bath of warm water, and finally transferred to warm funnels in an incubator at 42–45°. These simple expedients, which ensured that the rumen liquor remained continuously at rumen temperature or slightly above it, did in fact eliminate the abnormalities entirely, and showed that a slightly lowered temperature was an important factor in causing them.

Factors which favour the development of abnormal organisms

(a) *Lowered temperature at or near the time of adding glucose.* The following does not apply to rumen liquor obtained from concentrate-fed sheep (see later). After various trials it was found that rapid cooling of the rumen liquor in the funnel to 25°, then addition of glucose preferably dissolved in the minimum volume of cold distilled water, and replacement of the funnel in the 38° incubator, always gave a final yield of holotrich ciliates, after 2–3 hr., not far short of that expected; all the *Dasytricha* organisms then showed abnormality and so did a considerable proportion (up to 50 %) of the *Isotricha* organisms. It was never possible to bring down all the *Isotricha* organisms in an abnormal state. When the temperature was maintained at 25° throughout, before and after addition of glucose, the yield of organisms was much lower and the incidence of abnormality not significantly greater. The temperature of the funnel after replacement in the incubator of course remained below 30° for a considerable time, at least 45 min. It was not necessary to cool to 25° before adding glucose; the cooling could be carried out somewhat later. Thus, when glucose was added to strained rumen liquor at 32° the whole incubated for 20–60 min. (not longer) then rapidly cooled to 25° and finally re-incubated, quite as high an incidence of extreme abnormality was observed as with cooling immediately before the addition of glucose. Examination of the protozoa in the bottom layer withdrawn after 30 min. re-incubation following addition of glucose at 25° revealed that the organisms at this early stage were well filled with storage starch and only the initial stage of the abnormality was in evidence, viz. a slight withdrawal of the internal granular contents

away from the ectoplasm as in Pl. 1, fig. 2, organisms. It was repeatedly noticed that the holotrichs become more abnormal after they had settled at the bottom of the funnel, when allowed to remain there. Although fresh organisms were continually being deposited for several hours after addition of glucose, an examination of the holotrichs in the rumen liquor supernatant fluid at any given time (the organisms being collected by light centrifugation) revealed no more striking an incidence of abnormality than in the deposit which had collected by this time. It seemed unlikely, therefore, that there could be a preferential deposition of only slightly abnormal organisms in the early stages of incubation. Furthermore, motile abnormal organisms in a wet preparation examined at room temperature seemed consistently to appear more abnormal the longer they were examined.

Careful microscopic examination of freshly withdrawn rumen contents from hay-fed and concentrate-fed sheep did not reveal any holotrichs with the particular abnormality being investigated. This is consistent with the fact that the rumen temperature in a healthy animal is usually above rather than below body temperature (Krzywanek, 1929). It was found that the holotrichs *in vitro* required to be maintained at 25° or thereabouts for an appreciable time before abnormalities occurred, for when strained rumen liquor was rapidly cooled to 25°, glucose added, and the whole quickly warmed again to 37°, only a very small proportion of abnormal organisms was deposited on further incubation.

(b) *Necessity for the presence of a soluble metabolizable sugar.* Substitution of fructose or sucrose for glucose in the procedure outlined above under (a) gave a comparable deposition of abnormal organisms. Cellobiose, however, gave only abnormal *Dasytricha* organisms. The four sugars just mentioned were those found by Masson & Oxford (1951) to be convertible into storage starch by the holotrichs. The final concentration of glucose for production of abnormal organisms could be considerably less than 0.75%; even 0.1% yielded some completely abnormal forms.

Among the sugars found by Masson & Oxford (1951) to be not quickly convertible into storage starch, galactose, maltose, lactose and xylose were likewise found not to yield abnormal organisms of both genera. Because of the fermentability of these sugars by rumen bacteria with production of a scum of debris which would otherwise have sunk to the bottom, their use often facilitated the deposition of a clean layer of incompletely filled normal holotrichs. Galactose (1% or more) gave particularly good results in this respect and was used for obtaining holotrich suspensions for *in vitro* studies, the organisms in which could sometimes be rendered quite empty without undue mortality by overnight starvation in buffer. The holotrichs in rumen liquor were not harmed by long contact with any of these sugars at 25–32°. In one experiment with maltose the addition of glucose after 1 hr., by which time the temperature had risen to 32°, resulted in the later deposition of normal cells only.

The abnormalities could not be induced when an apparently insoluble metabolizable constituent (e.g. granular vegetable starch; see Sugden &

Oxford, 1952) was substituted for glucose. Rice starch, the grains of which are relatively small (see Kerr, 1950, p. 21, fig. 15), was swallowed with avidity by *Isotricha intestinalis*, and rather less readily by *I. prostoma* where the distribution at first was uneven, the grains being collected in that part of the endoplasm near the mouth. In neither species was any contraction of internal contents observed even when the strained rumen liquor (from sheep 8060 or sheep 1004) + starch in the funnel was kept at 35° for 45 min. before cooling (see section (a) above for corresponding experiment with glucose). The ciliates were more actively motile after ingesting rice starch than after metabolizing glucose. Since rice starch consists of a mixture of amylose and amylopectin, it is of course possible that amylose, the more readily soluble constituent, was preferentially attacked. The experiment was therefore repeated with waxy maize starch which is chemically similar to the protozoan storage starch, being purely amylopectin. Unfortunately the grains in this instance were mostly too large to be swallowed by *I. prostoma* (see Kerr, 1950, p. 19, fig. 9), but they were readily ingested by *I. intestinalis* without giving rise to any abnormality. Sugden & Oxford (1952) found that these holotrichs were apparently unable to utilize their own storage starch granules when the latter were supplied to them in suspension in a buffer. The holotrich starch preparation used had however been obtained from cells disintegrated by a detergent, and therefore might have contained traces of the latter. By using a more satisfactory preparation, obtained by mechanical disintegration, followed by purification by differential centrifugation, washing by decantation and finally dialysis, we now find that *Isotricha* sp. undoubtedly swallow the granules, becoming appreciably denser in appearance, although the distribution of the ingested granules is sometimes uneven. No well-marked contraction of the granules in a central zone was, however, observed.

(c) *Necessity for only a small initial content of storage polysaccharide.* Reproducible results with respect to abnormality in *Isotricha* organisms were not obtained with rumen liquors from concentrate-fed sheep no. 879 and 997, although *Dasytricha* organisms always became visibly abnormal in these instances after cooling and addition of glucose. This behaviour seemed to be due to the fact that only the *Isotricha* organisms can ingest and metabolize small vegetable starch grains (Sugden & Oxford, 1952). It was observed that many of the isotrichs deposited from such rumen liquors did in fact contain iodophilic granules of much larger size than their own storage grains. These larger granules were absent from the cells deposited from the rumen liquors of hay-fed sheep nos. 272 and 8060. Furthermore, the content of storage polysaccharide, with galactose-deposited cells, was visibly greater, on the whole, with isotrichs from concentrate-fed sheep than with those from sheep which had no, or very little, starch in the ration. *Dastricha ruminantium*, on the other hand, occurred in much the same relatively empty state in the rumen contents of all five sheep and was relatively less abundant in the concentrate-fed sheep. These observations help to explain the further fact that although it was always possible to harvest a good yield of holotrichs by addition of galactose to rumen contents of sheep 879, these preparations could not always be

satisfactorily starved *in vitro*, to yield cells sufficiently empty to give the abnormality by the method described in the next section.

An observation of possible significance was that the dominant *Isotricha* species in the concentrate-fed sheep was *I. intestinalis* which, as previously shown, ingests vegetable starch more readily than *I. prostoma*. This may account for the difficulty of obtaining from the concentrate-fed sheep rumen a suspension of *Isotricha* organisms, in which the majority of the cells showed a well-marked abnormality.

Attempts to reproduce the abnormal organisms in vitro: necessity for rumen liquor but not necessarily rumen bacteria

Reasonably empty holotrichs are obviously required for these experiments. Unfortunately 3 days of incubation in buffer were necessary before the organisms brought down by glucose were suitable, and by this time they were mostly moribund. Furthermore, when galactose was added to rumen contents from the sheep not fed concentrate the yields of holotrichs were liable to be excessively small. Sheep 8060 was the most useful one here. Fortunately it was occasionally possible to produce a marked occurrence of abnormal organisms particularly in *Dasytricha ruminantium*, in a bottom layer of buffer-starved normal holotrichs which had been initially obtained from the rumen contents of a concentrate-fed sheep by use of galactose. This was achieved by resuspending the organisms in partly or totally cleared rumen liquor freshly taken from a hay-fed sheep, cooling to 25°, adding glucose and re-incubating as previously described. In some instances the abnormal forms were observed even when the partly cleared rumen liquor had been heated at 90–100° for 2½ hr. in order to kill or at least inactivate most of its bacteria. On the other hand, when artificial buffer was used throughout as the suspending medium for the starved organisms, and when debris had been removed from the protozoan suspension by use of Nylon bolting cloth as previously described, numerous trials showed that it was practically impossible to produce the abnormality with starved suspensions, even in *Dasytricha ruminantium*. The presence or absence of calcium in the buffer seemed immaterial.

Survival of abnormal cells

Long-continued microscopic examination of living abnormal organisms never disclosed any tendency for spontaneous healing; the abnormality tended rather to get worse, as already stated. When washed suspensions containing a large proportion of abnormal organisms were kept in buffer overnight at 38°, the mortality of the abnormal forms was almost always apparently 100 %, even when the normal organisms survived satisfactorily. It was concluded that the abnormality is definitely harmful in the long run.

*Agencies which play no important part in the production of
these abnormalities*

At various times the factors listed below were considered as being involved in producing abnormalities of this kind but rejected for the reasons given:

Possible cause of abnormality	Reasons for rejection
Ingested rumen bacteria might be toxic	A washed suspension of mixed total rumen bacteria had no obvious effect when re-suspended in buffer, or in totally cleared rumen liquor
Ingested amylolytic bacteria might become toxic by attacking the holotrich storage starch	A pure culture of a saccharolytic and amylolytic rumen streptococcus (MacPherson, 1958) likewise had no obvious effect
Hyper- or hypotonicity in buffer, or unfavourable acidic pH value due to glucose fermentation	Abnormalities due to such causes do not in the least resemble those figured in Pl. 1.
Presence of pyrophosphate in the buffer used to dissolve the glucose, AnalaR K_2HPO_4 being unobtainable	Making up the buffer with AnalaR KH_2PO_4 and AnalaR KOH caused no change. The ciliates also tolerated added pyrophosphate quite well
Slower mutarotation of α -glucose (the ordinary crystalline form) at 25° as compared with 38°	Exactly the same result was obtained starting with crystalline β -glucose as with the ordinary α -variety
It is known that mannose is toxic to the holotrichs (Sugden & Oxford, 1952). Lowering of temperature might cause a slowing down of glucose fermentation to such an extent that a Lobry de Bruin transformation of glucose to fructose and mannose might take place within the cell	A careful microscopical examination of the early stages of the 'mannose' effect showed no close resemblance to the abnormalities due to glucose although an irregular distribution of cell contents was observed
Presence of traces of soap in the funnels left from previous scouring	A little household soap added to rumen liquor aided, rather than hindered, the deposition of normal cells, and did not cause any abnormalities to appear
Presence of bacterial fermentation products derived from glucose, but not usually present in rumen liquor as withdrawn from the rumen	Up to 0.01 N-lactic acid, succinic acid, ethanol, <i>n</i> -butanol, or acetone had no particular effect at pH 5.8.
Traces of possible products from bacterial amino acid degradation might be toxic	Effect is sometimes drastic (cf. Hogg & Elliott, 1951, on effect of indole on <i>Tetrahymena</i> sp.) but visible abnormalities in holotrichs did not in the least resemble those here studied.
Autolytic or other products from 'abnormal' ciliates might induce abnormality in others.	A finely ground suspension from a particularly abnormal holotrich culture had no effect in inducing abnormality when added to a suspension of normal cells.
Aeration of rumen liquor (during straining) may predispose the holotrichs to abnormality when subsequently fermenting glucose.	Quite vigorous pre-aeration of rumen liquor for 15 min. had surprisingly little effect when temperature control was maintained.

DISCUSSION

Five factors seem concerned in the production of these abnormalities and all must be acting in conjunction: (a) protozoan metabolism (considered in its widest sense) of glucose or other readily utilizable sugar which (b) must be dissolved in a buffer containing the soluble constituents, at least, of rumen liquor, the action taking place for (c) 30 min. or more, at (d) a temperature (25–30°) rather lower than the rumen normal, by (e) organisms which although motile and quite healthy in appearance initially, contain relatively little granular storage polysaccharide. The abnormality consists simply in an apparent alteration of the usual mode of storing newly synthesized reserve polysaccharide (i.e. amylopectin, Forsyth & Hirst, 1953). This new mode of storage, although drastically different from the normal, is not quickly lethal to the organism.

It remains to be discussed whether the alteration concerns the storage granules before or after they are made; in other words, whether it concerns starch synthesis or starch utilization (by autofermentation). We think that the weight of evidence points clearly to the last-named alternative. The abnormality does in fact get more pronounced after the virtual cessation of granule synthesis; its initial stages are often seen in organisms otherwise normally filled with storage granules; and most conclusive of all, the greatest incidence of abnormalities appears when the cooling to 25° occurs some 20–60 min. after the addition of glucose, during which period the organisms, acting at well above 30°, have stored much starch. When the cooling takes place after 90 min., no abnormalities develop. There is thus no evidence that the storage starch tends to be produced in a lump, rather than as separate granules, in a relatively empty organism which is metabolizing glucose at a somewhat lower temperature than usual. It appears rather that the characteristic change occurs after the granules are formed. As a working hypothesis we suggest that the abnormality is due to a profound disorganization of the enzyme system concerned with the autofermentation of storage starch. Heald & Oxford (1953) showed that this process, which finally leads to production of lactic, acetic and butyric acids, CO₂ and hydrogen, is not in abeyance when these organisms are actively metabolizing glucose. At present nothing is known about the mechanisms of synthesis and degradation of starch by these protozoa. Whatever the initial stages of the degradation may be they probably result in the production of soluble intermediate products of high molecular weight i.e. 'gums' which if allowed to accumulate might cause the granules to adhere to form a coherent mass when tightly packed together. It is clear from our results that the rate of production of storage granules is not greatly altered by a lowering of temperature.

French, Knapp & Pazur (1950), dealing with plant amylases, have shown that not only the rate of starch degradation, but also the degree of accumulation of intermediate products of high molecular weight, are dependent upon various conditions, including temperature. Without making any assumptions as to the nature of the protozoan 'amylases' we suggest that an abnormally

low temperature leads to a similar result with these holotrichs, namely that although the rate of production of the complete degradative enzyme system no longer keeps roughly in pace with starch synthesis, as it would at 38°, nevertheless the initial stage, i.e. the 'gum' production, still takes place.

We have no explanation however for two important facts: (i) inability to reproduce the phenomenon *in vitro* except in the presence of rumen liquor; (ii) the fact that the abnormalities cannot be produced at all by cooling if the ciliate has first ingested starch grains supplied extraneously in place of soluble carbohydrate. This is the case even when these granules consist of the holotrich storage starch itself. A detailed biochemical study of the 'amylase' of these holotrichs is clearly required in order to gain further insight into the cause of the abnormalities. This is now being carried out.

Our best thanks are due to the following colleagues: Dr J. W. Porteous for a supply of purified holotrich starch obtained by mechanical disintegration; the Photographic Department of this Institute for the photomicrographs and Mr M. Great for much technical assistance. We are also indebted to Mr J. Smiles of the National Institute for Medical Research, Mill Hill, for the agar method of 'fixation' of ciliates before photography.

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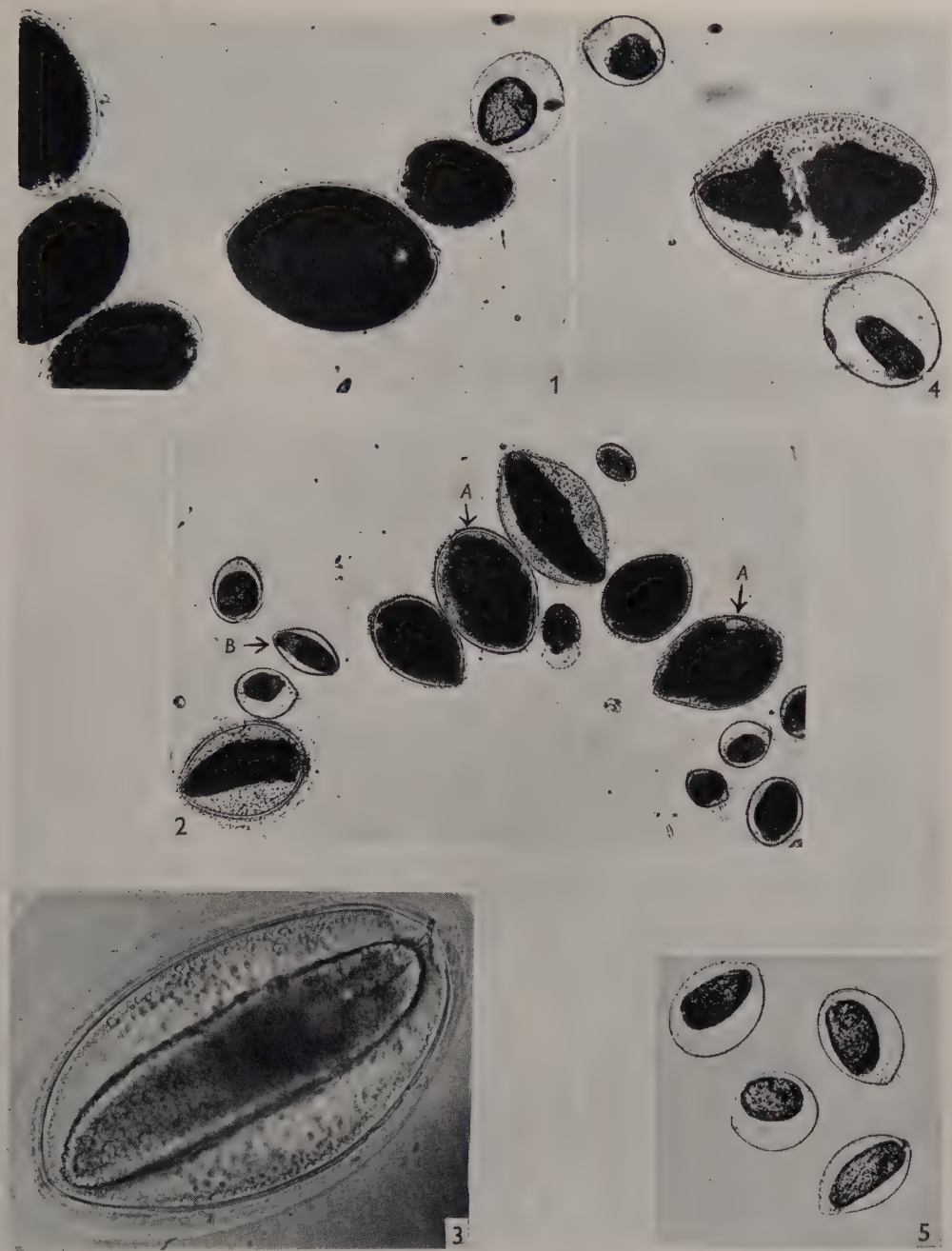
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EXPLANATION OF PLATE

All organisms photographed were alive and motile immediately before removal from the culture. They were rendered stationary by placing a coverslip with a small drop of culture on top of a smooth film of 0.2 % agar which was just on the point of solidification. The cilia do not show clearly because they were still in motion.

- Fig. 1. Four normal organisms of *Isotricha* spp. and one of *Dasytricha ruminantium* (to right of them), after glucose metabolism and filling of the organisms uniformly with storage polysaccharide granules. One highly abnormal *D. ruminantium* at extreme top right. $\times 152$.
- Fig. 2. Various stages of the abnormality in *Isotricha* spp. and *Dasytricha ruminantium*, the earliest being in organisms A and B respectively. Note numerous discrete granules still present in outer clearer endoplasmic zone of *Isotricha* spp. only. $\times 105$.
- Fig. 3. Usual appearance of the abnormality in an *Isotricha* organism with appearance of a simulated inner membrane which encloses the bulk of the storage polysaccharide. $\times 385$.
- Fig. 4. Later stages of the abnormality in an *Isotricha* organism (centre) and in two *Dasytricha ruminantium* organisms. $\times 153$.
- Fig. 5. Four abnormal *Dasytricha ruminantium* organisms. $\times 168$.

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J. MARGARET EADIE & A. E. OXFORD—ABNORMAL PROTOZOA. PLATE 1

(Facing p. 310)

CREWETHER, W. G. & McQUADE, A. B. (1955). *J. gen. Microbiol.* **12**, 311-313

The Intestinal Microflora of the Clothes Moth Larva *Tineola bisselliella* in relation to Wool Digestion

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SUMMARY: The intestines of clothes moth larvae, *Tineola bisselliella*, examined usually contained very few micro-organisms; this may be related to the high pH values of the intestinal contents. It was concluded that bacteria play no part in the digestion of wool by these larvae.

It was shown by Linderstrøm-Lang & Duspiva (1936) and by Waterhouse (1952) that the mid-gut of the clothes moth larva, *Tineola bisselliella*, has a reduction potential of *c.* -250 mV. at pH values near 10. These reducing conditions, thought to assist the digestion of keratin, might result from the metabolic processes of micro-organisms within the intestine. We therefore examined the intestinal microflora of the moth larvae.

METHODS

Larvae were obtained from laboratory cultures growing on unsterilized lactic casein powder (90-mesh) containing 10% dried yeast powder, and from naturally occurring colonies on greasy wool. Laboratory cultures on wool were obtained by transference of larvae or eggs from casein medium to greasy wool and incubation at the optimal temperature of 28°. Larval intestines were withdrawn aseptically after circular incision of the skin near the head and amputation of the tail tip.

Emulsions of four to six intestines in 0.05 ml. peptone water were equally distributed on six plates of nutrient agar containing 1% (w/v) glucose and 0.5% (w/v) yeast extract adjusted to pH values 7.5, 8.5 and 9.5. Plates were incubated aerobically and anaerobically at 28°.

RESULTS

Table 1 shows that with few exceptions the bacterial population in the gut, particularly in the mid- and hind-gut, was very small. This was confirmed by microscopic examination of smears of the gut contents which in general appeared to be free from bacteria. Smears of gut contents from larvae grown on wool consisted largely of partly degraded cortical cells and of other wool debris.

The larvae with high intestinal counts of bacteria (Table 1) were all derived from a single culture of larvae on casein medium and were shown by plating and by direct microscopic examination to be heavily infected with Gram-positive cocci. Other larval colonies on casein, however, were not infected in

this way. On transferring infected larvae to greasy wool the infection persisted indefinitely. In pure culture the coccus grew optimally at 28°, formed white colonies aerobically on nutrient agar, and anaerobically, slowly, a translucent growth. This coccus was catalase-positive, coagulase-negative (rabbit plasma), gave an acid clot in litmus milk, acid but no gas from glucose, produced no acetoin, and according to the classification of Shaw, Stitt & Cowan (1951) belonged to the *Staphylococcus lactis* group of organisms which are often found in dairy products.

Table 1. *Micro-organisms in the gut of Tineola larvae*

Source of larvae	Growth of micro-organisms	pH value of medium	Total count/gut
Casein culture (C.S.I.R.O. Geelong)	Aerobic	8.5	Many staphylococci
	Anaerobic	8.5	Many staphylococci
Casein culture (C.S.I.R.O. Canberra)	Aerobic	7.5	1
	Aerobic	8.5	120
	Aerobic	9.5	12
	Anaerobic	7.5	Spreader
	Anaerobic	8.5	0
	Anaerobic	9.5	1
Casein culture (Melbourne)	Aerobic	7.5	3
	Aerobic	8.5	1
	Aerobic	9.5	7
	Anaerobic	7.5	0
	Anaerobic	8.5	0
	Anaerobic	9.5	0
Wool culture (C.S.I.R.O. Canberra)	Aerobic	7.5	5
	Aerobic	8.5	7
	Aerobic	9.5	5
	Anaerobic	8.5	0
Wool culture (Melbourne)	Aerobic	7.5	Overgrown with fungi
	Aerobic	8.5	Overgrown with fungi
	Aerobic	9.5	Overgrown with fungi
	Anaerobic	7.5	0
	Anaerobic	8.5	1
	Anaerobic	9.5	40
Wild culture (greasy wool)	Aerobic	7.5	0
	Anaerobic	7.5	0

Only one colony developed (aerobically) on six plates inoculated with an emulsion of mid/lower guts from larvae on casein medium.

As the mid-gut has a pH value of about 10.0 (Duspiva, 1936; Linderstrøm-Lang & Duspiva, 1936; Waterhouse, 1952) the growth of the isolated coccus over a range of different initial pH values was compared with that of a culture having the same physiological properties but which was derived from a different source (supplied by the Department of Bacteriology, University of Melbourne). The organisms were incubated at 28° in a CO₂-free atmosphere in a medium containing: 1.0% (w/v) peptone (Difco); 0.1% (w/v) yeast extract (Difco); mineral salts; 0.15% Na₂HPO₄ (w/v) buffer. Both strains grew well in 24 hr. at initial pH values in the range 7.0–10.0; neither grew at pH 10.5. These results contrast with those of Dernby (1921), who recorded

a pH range of 5.6–8.1 for the growth of an organism called *Staphylococcus blanc* at an unspecified temperature. Beveridge (1940) showed that certain haemolytic staphylococci from milk were alkali tolerant.

CONCLUSION

The bacterial population in the gut of most of the *Tineola bisselliella* larvae studied was too small to influence the oxidation-reduction potential of the gut contents or to assist in the digestion of wool; it is concluded that bacteria play no essential part in this process. The high intestinal pH value is probably an important factor in the suppression of bacterial growth in the gut.

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THE PRINCIPLES OF MICROBIAL CLASSIFICATION

A REPORT OF THE DISCUSSION MEETING OF THE SOCIETY FOR GENERAL
MICROBIOLOGY, SEPTEMBER 1954

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Introduction: The Philosophy of Classification

By S. T. COWAN

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In the biological sciences, classification is a compromise between the dynamics of evolution and the stasis of the present; with microbes there is less apparent stasis, for with rapid multiplication there are greater opportunities for observing evolution in action. All classifications are subjective, and, like religious and political opinions, have a large element of aesthetic unreason about them. A classification consists of two elements: the objects to be classified, and the subject who feels the urge to classify them. There cannot be a classification without a classifier or taxonomist, and no two taxonomists

will approach objects in the same way. Thus a given set of objects may be classified in different ways by different taxonomists; even when the purpose is identical, the similarities may not be numerous. We cannot expect any particular classification to be permanent: Turrill (1952) has developed the idea of alpha to omega taxonomy, alpha classifications being the best that can be done with the information available; with increasing knowledge gaps are filled and errors corrected, and the classifications proceed along the Greek alphabet. Omega classifications are the ideals that never will and cannot be reached while evolution continues.

Why do we make classifications? If our intention is to pigeon-hole objects, all we need do is to give each a distinctive name or number, and arrange them in alphabetical or numerical order. But this is merely cataloguing, it is not grouping objects together, which is the essence of classification. Classifications may have a limited and specific purpose, as the water bacteriologists have classified coliform bacteria, or may be more generalized. General schemes, e.g. of Kingdoms, may be purely arbitrary in character and utilitarian in purpose, and may differ little from the dichotomous schemes used for identifications; on the other hand, they may attempt to be 'natural' and to show the relations of one group to another. The maker of a natural classification assumed that the creation of the world had been orderly and, by correlating the characters of his objects, he hoped to reconstruct the plan of the Creator. In a modern biological sense, a natural system is described as phylogenetic, and is supposed to reveal evolutionary trends, but we should remember that without an adequate fossil background much of our taxonomy is a mixture of speculation and wishful thinking.

Since classifications are largely subjective, their creation is an art, and the 'best' classifications are those made by artists with the keenest appreciation of what is both useful and intellectually satisfying. The classifier sorts objects so that the likes are brought together. If we knew everything about our objects we should be in a better position to make the sorting; in fact we must base our sorting on the known part. We can define a microbial classification as the sorting of micro-organisms in such a way that those with similar characters (or attributes) are brought together into units and those with dissimilar characters are separated and put into other units. The number of shared characters determines the homogeneity of the unit; with many characters shared the units will be small; with only a few characters in common, the association will be loose and the units large. The difference in unit size constitutes the basis for the ranks of hierarchical classifications.

Should we start at the top with big units and subdivide them, or should we work from the bottom, building small into larger units? Starting at the top we have, as primary units, algae, protozoa, fungi, bacteria, yeasts and viruses, and we divided them into subunits and sub-subunits. On the other hand, starting from the bottom, we can take the individual cell, the micro-organism, the colony, or the isolate, as the primary unit. The individual micro-organism may have one or more cells; theoretically such an organized whole makes the ideal unit, but as it is technically difficult to isolate and has a relatively short

life it is too ephemeral. A colony forms a population which has more substance and permanence, but may not be entirely homogeneous. In general an isolate is derived from a colony after technical procedures to ensure that it is 'pure', by which we mean that it is not composed of a mass of individuals showing widely divergent characters. It is convenient to make the isolate the smallest unit; similar isolates are collected together into the next larger group, the species.

Species concept. When biologists discuss classification they always come up against the species concept; for larger plants and animals the species is easier to define: Julian Huxley (1940) says that species are natural units, which (a) have a geographical distribution-area; (b) are self-perpetuating; (c) are morphologically distinguishable from other related groups; and (d) normally do not interbreed with related groups. Microbial species cannot be defined in this way; few microbes have a particular geographical distribution-area, though their economic effects may be localized, as in fungi of the order Uredinales in which host specialization plays an important part in the species concept (Ainsworth, 1954). Among algae, fungi and protozoa, species may be distinguished on morphological grounds, distinctions often emphasized by complicated life cycles. In bacteria gross morphology barely distinguishes higher ranks; cytology is useful at the generic level; at the species level the bacteriologist relies more on physiological than on morphological differences. Apart from certain protozoa and heterothallic fungi, most micro-organisms reproduce asexually so that interfertility is not a species character. In spite of this, satisfactory classifications can be made using other criteria, as in *Aspergillus* in which colony form and colour are combined with morphology. In microbiology we introduce characters not utilized by botanists and zoologists, such as nutritional requirements, metabolic and catabolic products, antigenic structure and pathogenicity; their use will be developed by other speakers.

The species concept is old, and originally was applied to any group of objects or ideas which had certain stated characteristics in common. We owe its use in the modern biological sense to the seventeenth-century English naturalist, John Ray. It was firmly established when Linnaeus grouped species into larger units, genera, which he also regarded as 'natural' units. We should remember that at this time naturalists believed that the species was preordained and the result of a divine creation; it was, therefore, unchanging. Linnaeus believed that there were as many species as there had been different forms created at the beginning of the world. Thus to the concept of a natural system was grafted an element of the supernatural (Heslop-Harrison, 1953).

In the seventeenth century John Locke wrote that 'the animal and vegetable kingdoms are so nearly joined, that...[when] we come to the lowest and most inorganic parts of matter, we shall find everywhere that the several species are linked together, and differ but in almost insensible degrees'. This is still true more than 250 years later. How, then, at the level of micro-organisms can we define a species? Topley & Wilson (1929) summed up the situation aptly in the sentence: 'The terms "genus" and "species", as applied

to bacteria, seem to us to defy definition, except as designations for two convenient groupings, of which the genus is the larger including group, and the species the smaller included group.'

A few examples will give a better idea of microbial species. Some protozoan species, such as *Entamoeba histolytica* and *E. coli*, are distinguished by essential differences associated with both the nucleus and the cytoplasm. Species of the *Aspergillus niger* series are distinguished by the dimensions of the primary sterigmata and of conidia, those of *Saccharomyces* by fermentation reactions. Among bacteria some species differ only in antigenic make-up, as in the salmonella group; in the genus *Clostridium* species are distinguished by the shape and position of the endospore, and by the toxicity of metabolic products. These examples show that the word species has different values in the different disciplines, and even in one it is used to describe different concepts in different genera. What is called a species of *Salmonella* would be a serotype of a streptococcal species, and the whole genus *Salmonella* is no more heterogeneous than the species *Escherichia coli*. Another example of the uncertain nature of a bacterial species can be found in the organism originally named *Salmonella arizona*, which was removed from the genus *Salmonella* because it fermented lactose. Other serologically related, but not identical, strains were found and an Arizona group or genus was formed: but not all these strains ferment lactose so that if we now apply the criterion upon which *Arizona* was separated from *Salmonella* the non-fermenters could legitimately be classified in the genus *Salmonella*, thus completing a circular argument.

The problem before us is to decide whether the species concept is tenable in microbiology, and if it is not, what we are to substitute for it. In the study of flowering plants, the older, or 'classical taxonomy' based on species is being supplemented by an 'experimental taxonomy' with new and diverse basic units, in which a general classification of the plant kingdom is not attempted (Heslop-Harrison, 1953). Compared with classical taxonomy, the experimental variety is essentially dynamic. Short generation times give microbiologists better chances to see evolution at work, and it is clear that the concept of static species must be abandoned in favour of something more elastic.

The nomenclatural type concept. In biological nomenclature the conception of nomenclatural types has developed, each type serving as a fixed point about which other isolates of similar characters can be grouped. These points are called nomenclatural types (Ainsworth & Cowan, 1954). Their value in classification depends on the validity of the taxonomic group (taxon) that each represents; if this is well founded, the type—of whatever rank—will, if it can be maintained without mutational change, be useful to the taxonomist. Does this type concept help us to classify microbes continually undergoing evolutionary changes? The types represent organisms at the time of their description and can be likened to genotypes; in the laboratory or under natural conditions the organisms may undergo changes, the characters of the changed organisms, or phenotypes, being so different from the original that they might be classified in different groups (or species).

Distinguishing characters. The choice of good distinguishing characters is

the art of taxonomy, and we should consider what qualities these 'good' characters possess. To the taxonomist, a good character is readily observable, and is not easily altered by changes in environment. For example, the typhoid bacillus has certain characters that are reliable because, in the natural state, variation from them is not observed: these characters are all negative and indicate lack of appropriate enzymes: they are the anaerogenic fermentation of sugars, the inability to ferment lactose, and the inability to produce indole from tryptophan. *In vitro* the last two characters can be changed by growth under special conditions, but the first is a character that, so far, has not been altered by the most devilish tricks of the students of bacterial variation. It is, therefore, a particularly good character, but so are the others, because in the wild state variation has not been found. In general, positive characters are regarded as more useful than negative ones, but most of them can be lost. Thus we may suppose that *Bacillus coli mutabile* is *Escherichia coli* with a partial loss of lactose-fermentation, and paracolons of the Ballerup-Bethesda series may be *E. coli* which have completely lost the enzyme lactase. The value of a positive character to the taxonomist depends on its regularity, thus the capacity to produce urease is a good character of *Proteus vulgaris* and a poor one for the Providence group, in which it is only sometimes present on first isolation and is quickly lost on subculture. Even a labile character may have value when the lability is regular, as the lactose- and sucrose-fermenting variants of *Shigella sonnei* (Cook, Knox & Tomlinson, 1951).

In different groups of organisms there is emphasis on different characters; morphology plays a greater part in the classification of algae and fungi than of bacteria, yeasts and viruses; serology is a bacteriologist's tool and is little used by workers in other disciplines. Microbiologists have great opportunities for borrowing techniques from each other: for example, serology can be applied to cultivatable protozoa or to fungi, but lack of familiarity with these techniques often obscures the obviousness of their application. The result is that in the characterization of micro-organisms there is a great unevenness of description, or great variation in quality of description. We need more fundamental knowledge, such as essential nutritional requirements, to supplement empirical observations. Other speakers will deal with the different criteria used in classification: all I stress is the inequality in value of different criteria in different groups of organisms. One of the newest approaches to the make-up of living organisms has been made by the geneticists, who have found in rapidly multiplying micro-organisms a happy hunting ground. Their evolutionary experimentation is showing how unrealistic is our species concept, for they find that certain transformations can be made almost as readily as a successful subculture.

Whither taxonomy? Where, taxonomically, will this lead to? Many workers look upon micro-organisms as bags of enzymes or protein molecules, and it is difficult to see how a system based on family relations can logically be applied to them. If we abandon our species concept, what alternative is offered? Mycologists have 'series' with an intermediate and undefined place between genus and species (see Baldacci, Spalla & Grein, 1954). Some serologists

subdivide a group of bacteria by antigenic structure (group in this case corresponding roughly to a genus); in some groups (e.g. *Salmonella*) they give each serotype a distinctive name so that the scheme has the superficial appearance of a Linnaean system, but in others (e.g. *Klebsiella*) second names are dispensed with, and species lose their identity. This system satisfies those experts who use it, but its inconsistencies do not attract the less specialised taxonomist.

In virology, the infective agents have been classified by the diseases they produce in susceptible hosts, and attempts are now being made to classify the viruses themselves. This cannot be done adequately until more is known of their nature and their properties, and it may be necessary to classify one group as living entities and another as complex proteins.

It is my purpose to sow doubt in your minds, and to leave you with a number of unanswered questions: (1) Does the hierarchical structure of a Linnaean system satisfy the requirements of microbiologists? (2) Can we accept the species concept, and all that this implies, or must we view our organisms as a huge spectrum composed of gradually merging forms? (3) How can we improve the descriptions which characterise our organisms so that the relations of one group to another can be more clearly seen? (4) Does the nomenclatural type play a useful part in building up a taxonomic scheme? (This, apart from its value in relation to nomenclature.) (5) Finally, are we wasting our time in trying to classify microbes?

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DISCUSSION

By J. W. HOWIE, *University of Glasgow*

It seems to me that bacteriologists would do well to learn from the modest attitude of virologists, most of whom have resolved that they do not yet know enough about viruses to classify them in a manner justifying the identification

of species and the use of Linnaean binomials. We are still without enough knowledge about a great many bacteria to know which of their characters are sufficiently stable and important to define genera and species. Our premature use of specific binomials has been repeatedly made ridiculous by swiftly changing ideas about classification. The resulting instability of nomenclature may be very stimulating to ourselves, but it is a constant source of irritation to those who are not greatly interested in systems of bacterial classification, but whose daily work is much concerned with bacteria. In medical work, for example, the typhoid bacillus has changed its name rather frequently with new ideas on classification and nomenclature. Within the past 20 years it has been *Bacillus typhosus*, *Eberthella typhosa*, *Bacterium typhosum* and *Salmonella typhi*. For the purposes of medical reporting it is best referred to by its common name: the typhoid bacillus. To assign the organisms to the genus *Salmonella* may be perfectly sound on grounds of immunology, but very confusing to a clinician to whom enteric fever caused by the typhoid and the paratyphoid bacilli means one thing and food-poisoning caused by one of the other salmonellas means quite a different clinical condition with an entirely different epidemiology. This is not a plea to abandon attempts at systematic classification but a suggestion that we should be modest enough about our tentative efforts to be content to use common, well-established names to designate familiar organisms to those who cannot reasonably be expected to be regular readers of the *International Bulletin of Bacteriological Nomenclature and Taxonomy*. Within the family of bacteriologists there is need for a great deal of work on bacterial systematics and for debate and discussion about valid criteria for classification; but until agreement on this subject rests on a much wider and sounder basis than at present, our constant although desirable changes of outlook should not be reflected in repeated alterations of the names that we use to describe bacteria to those whose interest is not primarily in the bacteria themselves, but in the important effects which they produce.

At present, our classification rests upon a largely subjective assessment of various attributes: morphological, cultural, biochemical, nutritional, immunological and ecological. Our knowledge of these attributes for any particular group of bacteria often depends more upon the special interests, aptitudes and facilities of those who have worked with the group than upon which of the attributes are useful and valid for classification. Our classifications often rest, therefore, upon partial knowledge acquired for purposes which have nothing to do with taxonomy. We should recognize these limitations by keeping our nomenclature on a basis of common names when we address our remarks to those who are not bacteriologists.

Dr Cowan has asked five questions, and I offer the following answers.

Question 1. Is the heirarchical system suited to bacterial classification?

Answer. Not in our present state of knowledge.

Question 2. Is the species concept of value in bacterial classification?

Answer. Not yet at any rate. Bacteria form something much more like a continuous spectrum than definable species.

Question 3. How can we improve our descriptions of bacteria?

Answer. By much work and sound thinking.

Question 4. Has the nomenclatural type a place in classification?

Answer. Yes—a very useful place if it remains stable and if it is what its label says it is.

Question 5. Are we wasting our time in attempting to classify bacteria?

Answer. Certainly not. We are making a very poor job of it only because we know too little. The attempt, however, is well worth while. Bacteria exist, so we must attempt to classify them. In any case, the exercise keeps Dr Cowan alive and happy.

By G. S. WILSON, *Public Health Laboratory Service, London*

It is difficult to draw up principles of classification of bacteria on any but arbitrary lines. The conception of species as held by Linnaeus was based on the belief in a fixed natural order in the world of living things. Experience, however, has shown that the demarcation of species is often far from clear, and that in many instances gradations can be traced between them. During the past 20 years or so the intensive study of bacterial variation has revealed in some groups, particularly the Enterobacteriaceae, so many intermediate types, not only between what were previously regarded as valid species, but even between different genera, that the task of defining species seems to be almost insuperable. To abandon the concept of species would be unfortunate, partly because it introduced order into the systematic study of bacteria, and partly because of the convenience it affords to descriptive bacteriology. In these circumstances it seems that the only practicable method of classifying and naming micro-organisms is to establish a series of nodal points along the continuous chain of variants, and to regard the organisms at these nodes, and for some distance on either side of them, as constituting species. The criteria used for selecting these nodes, and for determining the distance on either side of these nodes within which variants of the species might be included, will clearly need considerable discussion; but if this task were entrusted to a small group of bacteriologists of wide experience whose hands were not tied too rigidly by the present code of nomenclature, it should not prove impossible.

Nomenclature, the Handmaid of Classification

By G. C. AINSWORTH

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‘When I use a word’, Humpty Dumpty said in rather a scornful tone, ‘it means just what I choose it to mean—neither more nor less.’

‘The question is’, said Alice, ‘whether you *can* make words mean so many different things.’

‘The question is’, said Humpty Dumpty, ‘which is to be Master—that’s all.’

LEWIS CARROLL, *Through the Looking Glass*, 1871.

This intrusion is an attempt to remove a popular misconception; the rather prevalent notion that nomenclature takes precedence over taxonomy, that names in some way determine classification. ‘The question is’, as Humpty Dumpty once said when discussing semantics with Alice, ‘which is to be Master—that’s all.’ Codes of nomenclature merely regulate the selection and give precision to the use of the names employed after taxonomic decisions have been made and thus prevent the confusion that would result if every taxonomist showed the vigorous individualism of Humpty Dumpty, while at the same time they make it as easy as possible for a taxonomist to use a name so that others can understand just what he chooses it to mean—neither more nor less.

The best use of the short time available is possibly to consider a concrete example and the nomenclature of that well-known micro-organism, the ‘thrush fungus’, illustrates a number of basic nomenclatural principles and practices.

Since the thrush fungus was first given the Latin binomial *Oidium albicans* by Charles Robin in 1853 it has been classified in at least ten other genera, re-described as new at least thirty-six times, and in Lodder and Van Rij’s recent monograph (*The Yeasts*, 1952) ninety-two synonyms are listed after the name of their choice for this fungus. How is the selection of the scientific name for the thrush fungus made? One guiding principle of all international codes of nomenclature is that the correct name of a taxonomic group (or taxon) is the first legitimate one validly published with the same rank. For species this means the combination of a generic name with the earliest available legitimate specific epithet. As far as is known, the first specific epithet to be given to the thrush fungus was *albicans*, but before the binomial can be completed with a generic name taxonomic decisions must be made. How is this fungus best classified? The genus used by Robin, *Oidium*, is properly applied to the imperfect states of certain powdery mildews which are unrelated to the yeast-like thrush fungus. For a number of years medical men used the binomial *Monilia albicans* but the genus *Monilia* is correctly employed for the imperfect states of the fungi which cause brown rot of fruit. Since 1923 mycologists have favoured the classification of the thrush fungus in the genus

Candida of Berkhout and the binomial *Candida albicans* is now familiar. Unfortunately, there were technical objections to *Candida* as a name because the application of names is given precision by the use of nomenclatural types and there is uncertainty about Berkhout's intentions regarding the type of the genus *Candida*. Berkhout based her new genus on a fungus which she called *C. vulgaris* and which she believed to be that described by Bonorden in 1851 as *Oidium candidum*, but this is open to doubt. She certainly proposed *Candida vulgaris* as a new species but she may have intended *C. vulgaris* merely as a new name to avoid the illegitimate tautonym *C. candida*. The generic name *Candida* could, however, be rejected on another score. By including the thrush fungus in the genus *Candida*, Berkhout included an earlier and overlooked generic name, that of *Syringospora*, the name given to a genus specially proposed by Quinquand in 1868 for the thrush fungus. Under the Botanical Code, *Syringospora* takes precedence over *Candida* and the correct name of the thrush fungus when classified according to Berkhout's views is *Syringospora albicans*. But the revival of the generic name *Syringospora* would upset a familiar and widely used name for this common pathogen and so the conservation procedure was invoked and the International Botanical Congress held in Paris in July 1954 agreed that *Candida* should be conserved against *Syringospora* and the application of *Candida* can be clarified by accepting Berkhout's description of her culture as the type of *C. vulgaris* (now considered to be a synonym of *C. tropicalis*), the nomenclatural type of the genus. *C. albicans* (Robin) Berkhout is now a correct name under the Code for the thrush fungus. It need not be the only correct name and it need not be the name for all time but it is the name which reflects current taxonomic opinion. How many synonyms there are of *C. albicans* is again a matter of taxonomic opinion. A taxonomist may at any time consider that Lodder and Van Rij were mistaken in considering a particular species indistinguishable from *C. albicans* when the species in question would be restored as an independent taxon and its name removed from the synonymy of *C. albicans*.

This example indicates some of the mechanisms of name selection and the moral to be drawn is that good taxonomy is the basic requirement if nomenclatural confusion is to be avoided.

The various international codes of nomenclature are not penal codes. They reflect current usage and they are slowly modified as practice shows them to be inconvenient or to lead to confusion rather than order in the naming of living organisms. The Codes thus grow with the growth of taxonomic practice and attention may be drawn to the fact that the codes have been developed to meet the requirements of classical taxonomy which for larger organisms and for many micro-organisms is firmly based on morphology. For the taxonomy of micro-organisms morphology must frequently be supplemented if not replaced by physiological, biochemical, or serological characters and for the naming of certain taxa defined by such criteria codes of nomenclature as yet offer little guidance.

General Morphology

By T. GIBSON

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The morphological characters of the bacteria, while they are useful or indispensable in the recognition of many forms, have various limitations as aids in taxonomy. Indefinite distinctions and the occurrence of variation, either mutational or in response to the conditions of culture, are important limitations. The custom of giving to morphology the premier position among the attributes used in classification has created many problems.

In the Eubacteriales the form of the cell fails to provide a good separation of *Streptococcus* and *Lactobacillus* and of *Staphylococcus* and corynebacteria; cell grouping has proved unsatisfactory for distinguishing *Sarcina* and *Gaffkya* from other cocci. The question whether the position of flagella on the cell surface is a satisfactory basis of classification still remains to be clarified. The presence or absence of flagella is rarely a useful feature; motility has been detected in a few species or strains in several genera of the Gram-positive and typically non-motile bacteria (*Staphylococcus*, *Sarcina*, *Streptococcus*, *Lactobacillus*, *Corynebacterium*). The formation of heat-resistant endospores has continued to be a satisfactory taxonomic character, but its usefulness is almost confined to the segregation of the groups *Bacillus* and *Clostridium*.

The order Actinomycetales, which appears to offer wide scope for the successful utilization of morphology, presents many taxonomic perplexities. Here, generic boundaries are obscured by the transitional forms which may be isolated from natural habitats and by the stable variants which may be obtained from pure cultures. Most of the organisms in which a branched mycelial structure is a conspicuous feature are now allotted to the genera *Streptomyces*, *Micromonospora*, *Nocardia* and *Actinomyces*. This classification, which is largely morphological, is admittedly imperfect, but there is fairly general agreement that no better taxonomy is in sight. The finding that there is a smooth transition in morphology from the fungus-like *Streptomyces* to the structurally simpler corynebacteria and mycobacteria raises the problem of where to draw the line between the eubacteria and the actinomycetes.

The Myxobacteriales, in which segregation and division is based mainly on morphology, show two serious taxonomic weaknesses. First, the more complex forms of fruiting body have been detected only in natural materials where their variability cannot be assessed. Secondly, the separation of *Cytophaga* from the eubacteria has come under suspicion with the isolation from soil of organisms which appear to be identical with typical cytophagas except in a failure to show gliding motility on the surface of agar media.

It may be concluded that there are few definite boundaries separating the distinctive forms of bacteria, and that morphological characters tend to lack the precision desirable in determinative keys. Nevertheless, these conclusions need not detract from the great value of the microscope as a tool for the recognition of bacteria.

The Value of Cytological Studies in Elucidating Natural Relationships among Bacteria

By K. A. BISSET

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The nature of the ancestral bacterium is the fundamental problem in any evolutionary system, and this is a point upon which cytological evidence can be of considerable assistance. The classification of *Bergey's Manual* (1948) ignores this question almost completely, and confines itself to the suggestion that bacteria and blue-green algae are closely related. *En passant*, it may be remarked that the blue-green algae are perhaps the sole group in which no immediate relationship with the bacteria can be found because, alone of all living creatures, they show no evidence of ever having possessed flagella.

The evolutionary scheme of Kluyver & van Niel (1936) commences with the assumption that the cocci are the ancestral group, because they alone have 'retained the primitive morphology'. It is, of course, tempting to believe that an apparently simple, spherical form is primitive, but a very small acquaintance with the cytology of cocci renders it apparent that their ostensible simplicity of form is exceedingly deceptive, and that they frequently possess a complex, septate structure, suggesting a relationship with the more highly evolved, septate Gram-positive bacilli. There are other serious objections to the scheme of Kluyver & van Niel, notably that it requires an independent, multiple origin for flagella, endospores and Gram-positivity, and that the relationship of the 'primitive' coccus to other forms of life is entirely obscure.

In order to elucidate the problem of the ancestral bacterium from the cytological viewpoint, it is necessary to consider what are the cytological characteristics of bacteria as a group. Obviously, not all bacteria possess all, even of the most typical of these, in their most typical form, but there exist certain peculiarities which distinguish bacteria from other micro-organisms, and those forms in which one or other of these is absent are usually found to be quite closely related to others in which they are clearly recognisable.

The vegetative nucleus of bacteria is in the form of paired rodlets, dividing reductionally, and lying at right angles to the long axis of the cell. The resting nucleus is vesicular with an eccentric granule (cf. Bisset, 1950*a*). The flagella are monofibrillar. Divisions between adjacent cells contain an element of the cell-wall proper; the wall itself is slightly spiral (Pijper, 1946). There are, in addition, certain peculiarities of cytogenetic behaviour which are beyond the scope of this discussion.

The nature of their septation and the possession of flagella serve unequivocally to distinguish bacteria from blue-green algae, which bear a superficial resemblance to filamentous bacteria. Similarly, the resemblance of *Streptomyces*

spp. to the asexual phase of certain ascomycetes, with which they have been compared, does not extend their nuclear structures, which are typically bacterial (Klieneberger-Nobel, 1947). In addition, an entire series of forms connects the 'higher bacteria' with their lower relatives.

If, therefore, bacteria are neither ascended from blue-green algae nor descended from moulds, much less arisen *de novo* from a simple, spherical cell, it is necessary to examine their internal relationships in order to discover a clue to their external derivation, and hence to the direction in which they may have evolved.

The most important clue in the list of characters given above is the observation that the clear-cut distinction formerly drawn between spiral and rod-shaped bacteria for taxonomic purposes is not justifiable. The great majority of bacteria are, in fact, more or less spiral rods. Such exceptions as cocci or branched filaments have obvious affinities with more typical genera.

There is thus a complete series of forms between the markedly spiral, aquatic bacteria with polar flagella, and the much less spiral rods, common in soil but rarely and apparently accidentally found in water, which are either devoid of flagella or have a very large number arranged peritrichously. Although the latter are often described as actively motile, this usually means that they exhibit the power of swarming over a moist, solid surface, or in a viscous medium. A vibrio with a single polar flagellum swims much more efficiently than, for example, *Proteus*.

It thus appears highly probable that peritrichous flagellation and complete loss of flagella are alike adaptations of a fundamentally aquatic organism to a terrestrial environment. The alternative explanation, that the terrestrial bacteria have colonized the water, and evolved spirillar types via pseudomonads from bacilli, postulates a separate origin for bacterial flagella, and runs contrary to all experience in other groups of organisms. The return of a terrestrial form, once evolved, to an aquatic environment may occur in any group, and there is evidence of it also in bacteria, but the main trend is from aquatic to terrestrial in every case.

The series of intermediate forms between a spirillum and a bacillus is so obvious that the scheme of Kluyver & van Niel also shows exactly this relationship, but attempts to derive the two ends of this chain independently from the 'ancestral' coccus, by suggesting that Gram-negative bacteria and pseudomonads are each more closely related to staphylococci than to one another. Even the most general acquaintance with bacterial morphology would render this proposition unattractive. The similar morphological series between the more and the less complex branched Gram-positive bacteria is also apparent, although their exact relationship with the Gram-positive spirilla is obscure.

The second phase in the process of evolution away from an aquatic environment usually consists in an exploitation of the potentialities of the atmosphere as a distributive agent, and this process can be observed in bacteria. Such obvious examples as the aerial hyphae and conidia of streptomyces hardly require emphasis, but a parallel mechanism is found in the stalked fruiting

bodies of the higher myxobacteria, the vegetative swarms of which are capable of a crawling motion on solid surfaces. Further types of adaptation to aerial distribution are found among the septate Gram-positive bacilli and their relatives. Perhaps the most remarkable is the endospore, that uniquely resistant stage in the life cycle of the Bacillaceae (Bisset, 1950*b*).

It is not suggested that all aquatic bacteria are necessarily primitive. A high degree of adaptive specialization to this environment is exhibited by the sessile caulobacteria and chlamydobacteria, but both retain typically primitive characters in the motile distributive stages, which may resemble pseudomonads, vibrios, or even spirilla, and which indicate that these groups are quite closely related to the main line of bacterial evolution (Bisset & Grace, 1954).

In general, the possession of flagella must be considered a primitive character, and the flagellate bacteria, together with their nearer relatives which may have lost their flagella at a relatively late stage of evolution, compose the order Eubacteriales. This definition includes a variety of caulobacteria, chlamydobacteria and others, especially autotrophs, which were until recently excluded from this order. Within the Eubacteriales, the most important subdivision is between the septate Gram-positive and the unicellular Gram-negative forms. In the classification I have proposed (Bisset, 1952) these are accorded the status of suborders, Bacillineae and Bacteriineae respectively. The true caulobacteria and the flagellated true spirochaetes are also granted subordinal rank, but the spirilla, pseudomonads and chlamydobacteria are all included with the typical Gram-negative bacteria in the suborder Bacteriineae. The Bacillineae comprises the true cocci and the lactic acid bacteria with the sporing bacilli. This suborder, however, has marked affinities with the order Actinomycetales (not including the true streptomyces which in some quarters continue to be mis-called actinomycetes). Like these they are septate and Gram-positive, and many of the genera are entirely non-motile, but they are distinct in that their septation and cell-division is symmetrical, and in that they do not form lateral buds or branches, whereas the members of the Actinomycetales are irregularly septate and bud or branch more or less freely. In such circumstances the allocation of the suborder Bacillineae to one order or the other can only be an arbitrary decision. Almost certainly its members have a close affinity with the ancestors of the Actinomycetales and Streptomycetales, but their frequent possession of flagella cannot be ignored.

To return to the question of the origin of the simpler branched bacteria, it has been indicated that the streptomyces and the sporing bacilli resemble one another closely in respect of their nuclear cytology, and it is not beyond the bounds of possibility that some at least of the Actinomycetales may be descended via a more complex, branched sporogenous form, perhaps a true streptomyces, from the Gram-positive eubacteria. Certainly the nuclear behaviour of the ostensibly simpler members of the order is less typically bacterial than that of the more complex; so far, at least, as can be discerned.

At the same time, it must be confessed that the Actinomycetales constitute

the order in which the least confidence of a common phylogeny can be placed. As always, much more information is required before firm opinions can reasonably be expressed, but an example of the sort of degenerative process which may be anticipated, and upon which cytology almost alone can shed a little light, is given by the description by Prévot (1953) of a system of temporary branching and of the maturation of the resting cell in a member of the Fusiformis group, which Prévot believes to be related to the anaerobic actinomyces. The cytological detail bears an astonishing resemblance to portions of the cycle in *Actinomyces bovis* described quite independently by Morris (1951). It is exceedingly improbable that the complex cycle in *A. bovis*, culminating in what appears to be a vestigial remnant of a process once designed to produce an aerial spore, could have been evolved in such delicate, anaerobic parasites as are both *A. bovis* and Fusiformis, but it is more than likely that they represent stages in the degeneracy of such parasites from a free-living, freely sporing ancestor. And to this extent the theory that the simpler bacteria are degraded from mould-like forms may have its share of truth. Comparable examples of functional degeneracy accompanying the adoption of a parasitic mode of life are probably afforded by *Clostridium* spp., *Bacillus polymyxa* and *Rhizobium* spp., and almost certainly the apparent extreme simplicity of structure of such obligate parasites as *Brucella*, *Pasteurella* or *Haemophilus* spp. is not primitive (Bisset, 1952).

On the other hand, certain morphological types of bacteria, notably pseudomonads, are endowed with a degree of versatility which enables them to adopt almost any mode of life, without undergoing modification.

One of the most encouraging features of this application of more refined morphological principles to the study of bacterial systematics is that the modifications which it imposes upon existing systems are almost without exception simplifications. Definitions of groups are especially simplified. A second encouraging feature is the manner in which evidence from such sources as biochemistry or genetics can be fitted into the same framework. But most encouraging of all is the reasonable explanation which it offers for so many hitherto confusing and enigmatic observations in the biology of bacteria.

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The Impact of Genetics

By G. PONTECORVO

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So far there has been no impact of genetics on microbial systematics. There has been a very fruitful one, of course, on the systematics of higher organisms. What is unwisely called 'The New Systematics' is the result of the recent introduction of evolutionary and genetic approaches into a systematics already well developed in a large number of groups of animals and plants.

The impact of genetics and evolution on microbial classification is bound to come. It will start as soon as the systematics of one or more groups of micro-organisms is sufficiently advanced: my guess is that the yeasts, the coliform bacteria, and the filamentous ascomycetes are going to be the most likely victims.

Classical systematics is one in which Linnaeus's idea that there are as many species as were created in the beginning is unconsciously still the master. Intraspecific variation is resented, let alone variation in time: it is a nuisance which interferes with classification. The 'new' systematics accepts enthusiastically variation at both these levels, and even makes use of it in its deductions.

Before I go into this matter, let me again emphasize: until the description of the forms of a particular group of organisms is well advanced, there is no question of 'new' systematics. It is estimated that over 90 % of the living species of birds have been identified. In certain families of mammals, Lepidoptera, flowering plants and ferns the 'old' systematics is almost as advanced as this. In all these groups the first step in classification ('A species is a systematic unit considered as a species by a competent systematist') could be followed by one based on biometric, ecologic, genetic and cytologic analysis. Perhaps in about half a dozen groups of higher organisms this four-prong attack has begun. In micro-organisms no group is ripe as yet.

Now the contribution of genetics to the systematics of higher organisms has been mainly the following. Every organism is different from every other one. Many of these differences are due to differences in hereditary determinants like the genes, whose mechanism of transmission is fully understood. Others are due to differences in other transmissible determinants, whose nature and mode of transmission is unknown. A third kind of difference is untransmissible, such as age and most effects of the environment.

A dynamic definition of a species is the aggregate of individuals which, directly or indirectly, can contribute over an indefinite number of generations to a common pool of hereditary determinants. For instance, a male rabbit cannot pool its genes directly with another male rabbit, but it can do so through its progeny. In the simplest situation, a species includes all the potentially interbreeding individuals, the descendants of which are also potentially interbreeding.

The criterion for classifying an individual into a species is that of its potential contribution to, or its sharing in, the gene pool of that species. Because of the nature of the hereditary process, the transmissible variation which is expressed and detectable in any one population is always a very minor part of its potential variation. The relative proportions of the two vary, even between populations of one species, with the environment and with the 'genetic system'. The 'genetic system' includes the mating structure of populations (inbreeding, sex differentiation, etc.), the details of the hereditary processes, the way in which the gene pool is distributed throughout the species, etc. All this varies tremendously both within and between species. A knowledge of the genetic system makes sense of the otherwise impossible systematics of *Rubus* or *Primula*, to give two examples from plants, and man or *Drosophila*, to give two examples from animals.

In conclusion, there is great individual variation within a species and to distinguish individuals of one species from those of a closely related one we use the yardstick of gene flow versus gene isolation. I hasten to repeat that even in the systematics of higher organisms this criterion is barely beginning to be used, and that for a long time in many groups it will have to be used only as an inference.

Now as to micro-organisms. I repeat that the systematics of no group is as yet ripe for the 'impact'. Even in the best described groups the proportion of forms described is very small. There is still an enormous amount of useful work to be done of a purely descriptive type, followed by as rough and as practical a classification as 'old' systematists can devise.

When the next stage will be reached we shall have some most interesting special problems set by micro-organisms as to their genetic systems. Besides the standard processes of sexual reproduction the transmission of hereditary determinants in micro-organisms takes a variety of novel forms. Already several of them have been discovered in barely 10 years of work: (a) particulate cytoplasmic inheritance, such as that of Kappa in *Paramecium* and probably of cytochrome oxydase in yeast; (b) non-particulate cytoplasmic inheritance such as that of antigens in *Paramecium*; (c) transformation in pneumococci; (d) 'transduction' in *Escherichia coli* and *Salmonella* spp., besides what seems to be a proper sexual cycle in *Escherichia coli*; (e) heterokaryosis in filamentous fungi; (f) the 'parasexual' cycle in filamentous fungi; (g) recombination in coli-phages and in various animal viruses.

Clearly, even though most micro-organisms may be asexual—and we should not be too sure about that—they can cope otherwise with the necessity for pooling hereditary determinants. Even if the discovery of new processes of transmission and recombination of hereditary determinants continued at the present rate this should not prevent the introduction into microbial 'new systematics' of some criterion involving the idea of common ancestry and potentially common posterity. This has been the main contribution of genetic and evolutionary study to systematics in higher organisms and its stimulus has been very powerful. I am confident that it will be equally fertile in microbial systematics, where the difficulty of rapid variation in time is so much more telling than in higher organisms.

Considerations of General Physiology

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Whilst morphological characters form the basis of the accepted systems of classification of bacteria, from the time of Orla-Jensen, attempts have been made to devise systems based primarily on the physiology or the biochemical modes of life of these organisms. This line of thought has had a permanent effect. An examination of the accepted generic names shows the influence of this approach; thus we have *Propionibacterium*, *Desulphovibrio*, *Thiobacillus*, *Nitrosomonas*, to instance but a few examples. At one time it was considered by many that certain types of physiology were not only useful aids to classification but had phylogenetic significance. In particular it was considered that autotrophic bacteria were primitive organisms and that increasing nutritional complexity indicated more highly evolved forms. The powerful arguments put forward by Haldane and by Oparin have made this hypothesis untenable and the question of the significance, if any, which can be attached to physiological properties has to be reconsidered.

The last few years have seen an immense improvement in the methods available for the separation and analysis of macromolecules of biological origin and in the methods of detection and estimation of cell constituents of low molecular weight. Application of these methods is still in its infancy, but sufficient work has been done already to suggest that the same structural pattern is found in all living things. Thus all cells so far examined appear to contain proteins, ribose nucleic acid and deoxyribose nucleic acid, all of which have the same basic structure and all of which are built up from the same components; the differences are differences in detail only. Likewise the same coenzymes seem to function in all cells. It is not surprising, therefore, that the synthesis of individual components of the cell proceed by mechanisms common to all cells in which the particular component is found. It is a fact that so far only very few synthetic pathways have been studied in detail, but wherever comparative studies have been made the answer is always the same. Thus, the mechanism of arginine synthesis is the same in animal tissues, *Neurospora* and *Escherichia coli*. Likewise the synthesis of serine appears to be similar in animal tissues and in bacteria and, more recently, studies of the synthesis of porphyrins have shown that the same set of reactions occurs in both animal tissues and in a variety of bacteria. Despite the enormity of the gaps in our knowledge I feel fairly confident that a given type of compound will be synthesized by the same mechanism in all organisms capable of achieving the synthesis of that compound. If this be so it is clear that, for the present at least, we cannot obtain any assistance in the building of a phylogenetic system of classification of bacteria from studies of synthetic mechanisms.

The nitrogen requirements for growth are met either by elementary nitrogen or by nitrogen in some combined form. The ability to fix gaseous nitrogen is distributed in what appears to be an irrational way. Thus *Azotobacter* spp., various *Clostridium* spp., all the photosynthetic bacteria, the rhizobium-leguminous plant complex and certain blue-green algae fix nitrogen, and it is difficult to believe that these forms bear any close relationship to one another.

Carbon requirements are satisfied either by carbon dioxide, as in the case of the autotrophic bacteria or by carbon in organic combination in the case of the heterotrophs. Organisms are known which will grow upon oxalate and on formate, and in these cases it is impossible to decide at present whether the use of such oxidized forms of carbon is a manifestation of the autotrophic mode of life or whether the organisms concerned are heterotrophs. The ability to use carbon dioxide for growth is a property shared exclusively by the autotrophs and the green plants. On the other hand, the utilization of carbon dioxide for the synthesis of individual compounds, for example aspartic acid, is common to plants, bacteria and animals. This universal ability to fix carbon dioxide has tended to obscure the importance of the autotrophic bacteria, but the essential feature of the autotrophs is that, like the green plants, they satisfy all their requirements for carbon with carbon dioxide.

Recent work on the fixation of carbon dioxide in photosynthesis has shown that the mechanism is quite different from any previously described. We do not know how carbon dioxide is fixed in the chemosynthetic autotroph but, in the photosynthetic autotroph the mechanism seems to be the same as that found in the green plants; namely, one which involves the formation of 3-phosphoglyceric acid from a 2-carbon compound and carbon dioxide. Just how unique this mechanism is, is not known but, considering all the work which has been done of late on carbon dioxide fixation, it is perhaps significant that the formation of phosphoglyceric acid by carbon dioxide fixation has only been recorded in photosynthetic systems. Phosphoglyceric acid is such a stable substance and so easy to isolate that it would be difficult to overlook. It is important that the phosphoglyceric acid mechanism should be looked for not only in the chemosynthetic autotrophs but also in the heterotrophs and in animal tissues. Should it prove to be peculiar to the autotrophs and the green plants we would have information which might be of considerable phylogenetic significance.

Whilst the ability to metabolize a given compound is frequently used in the differentiation of two otherwise similar organisms no one would go so far as to claim that a close relationship existed between all organisms which metabolize a given compound. Such a conclusion would bring together all organisms which utilize benzoate and such a group would include certain strains of *Azotobacter*, *Mycobacterium* and *Pseudomonas*. Likewise, van Niel has indicated the difficulties which would ensue were all the organisms which utilize hydrogen sulphide grouped together; for this would mean the inclusion of such morphologically diverse organisms as *Beggiatoa*, *Thiovulum*, *Thiobacillus* and *Chromatium*; and, because of the similarity of the pigments, the *Athiorhodaceae*.

Basically two main energy sources are exploited for growth, viz. chemical

energy and radiant energy. In the former instance chemical energy is ultimately made available by oxidation-reduction reactions and there are two types of process. First, there is the formation of high energy phosphate bond compounds at the substrate level. That is to say, at certain points along the metabolic chain phosphorylated compounds are produced which, in the presence of the appropriate enzyme, phosphorylate adenosine diphosphate giving adenosine triphosphate and the dephosphorylated intermediary. In the second method phosphorylation of adenosine diphosphate is coupled with the transfer of electrons from reduced pyridine nucleotides to oxygen via the cytochrome system. Whilst the mechanism of this process is not known, it is clear that the development of this system is of considerable evolutionary significance, increasing as it does the amount of energy which is available from a given substrate under aerobic conditions. Organisms which use oxygen as the first acceptor are termed aerobic and those which use other acceptors in the oxidation-reduction reactions are described as anaerobic. The distinction is a biochemical one but the terms are not always used in this sense. The terms anaerobe and anaerobic tend to imply a sensitivity to oxygen rather than the inability to use oxygen as a terminal acceptor. It would be preferable to define anaerobes as organisms with an anaerobic metabolism and aerobes as organisms with an aerobic metabolism; in this way the fundamental differences between the two groups of organisms are made clear. To differentiate by means of the trivial character of sensitivity to oxygen, which is difficult to define, is not helpful.

The phylogenetic significance of these two characters is doubtful. Organisms with an anaerobic metabolism are found in most families and it is probable that the ability to synthesize the cytochrome system can be lost without unduly jeopardizing an organism's chances of survival. Whilst there is evidence that the atmosphere of the earth was, for a considerable time, devoid of oxygen, there is no reason for believing that the present-day anaerobes are primitive organisms.

The utilization of radiant energy for growth is, in all cases, associated with the presence of a chlorophyll type of pigment; though chlorophyll is not in all cases the most active pigment. The various chlorophylls which have been described differ only slightly from one another in chemical structure and from what has been said above it is reasonable to believe that the mechanism of synthesis of these various chlorophylls will be essentially the same in all organisms. This raises the question of whether such a mechanism could have evolved independently in a number of unrelated organisms or whether it evolved once only. If the latter be accepted then, clearly, all organisms which contain chlorophyll must have descended from a common stock and chlorophyll can be taken as a useful indicator of phylogenetic relationship. On the other hand, if chlorophyll formation be an instance of parallel evolution then there is little more to be said.

In the animal kingdom creatine phosphate is found only in vertebrate muscle, in *Balanoglossus*, one of the Protochordata, and in the Echinoidea which are of course invertebrates. It is of interest that morphological and

embryological evidence suggests that these organisms are related, and that the biochemical evidence supports this view. Perhaps chlorophyll is a biochemical indicator of phylogeny in much the same way as is creatine phosphate. Pringsheim has recently made a similar suggestion. The recently discovered diaminopimelic acid and related compounds may also be of use as indicators.

Haemoglobin is characteristic of vertebrate blood and occurs sporadically in the invertebrates. Until recently there was a tendency to believe that haemoglobin was peculiar to the animal kingdom. Recently, however, it has been found to occur in the nodules of leguminous plants and in yeast cells, and these observations might lead one to doubt the argument outlined above, namely, that possession of a particular compound indicates a common origin. However, I do not believe that this criticism is valid. Haemoglobin consists of a protein coupled with haem and there is a wide range of such compounds not all of which have the property of combining reversibly with oxygen; the enzymes catalase and peroxidase are good examples of this. The nature of the protein to which the haem is coupled seems to condition the properties of the resulting compound. It is thus not difficult to imagine haemoglobins cropping up in a wide variety of organisms purely by chance, for all that is required is a slight alteration in the protein synthesizing mechanism to obtain a protein which, when coupled with haem, produces a compound which can combine reversibly with oxygen. On the other hand, the synthesis of a molecule as complex as chlorophyll must involve the formation of a large number of enzymes whose actions must be co-ordinated, and the probability that such a system could have evolved more than once appears to me to be remote.

If it be accepted that all the chlorophyll-containing organisms have a common origin then it follows that the Athiorhodaceae, the Thiorhodaceae, the Chlorobacteriaceae and the green plants must have evolved from the same stock. Nothing is known of the detailed chemistry of the chlorophyll of the Chlorobacteriaceae; on the other hand, the chlorophylls found in the two groups of purple bacteria appear to be identical. In addition, both the Athiorhodaceae and the Thiorhodaceae have many morphological characters in common with the Pseudomonadaceae; and the Athiorhodaceae when grown aerobically in the dark cannot be easily distinguished from this group. The position of the Chlorobacteriaceae is not so clear-cut; their chlorophyll appears to differ from that of the other photosynthetic bacteria and their morphology is also quite distinct, for they are Gram-negative non-motile cocci. At the moment the most simple interpretation would appear to be that they are representatives of a line of evolution different from that of the Thiorhodaceae and the Athiorhodaceae.

The obvious morphological similarities between the two groups of purple bacteria on the one hand and the Pseudomonadaceae on the other suggest that they all belong to the same line of evolution. It may be a coincidence that most of the chemosynthetic autotrophic bacteria are characterized by being Gram-negative and with polar flagella when motile.

In view of this it is not too unreasonable to suggest that all the Gram-

negative organisms with polar flagella have evolved from the same stock as the green plants in the following way. The photosynthetic bacteria have retained the ability to utilize radiant energy and carbon dioxide for growth. The autotrophic bacteria have retained only their ability to utilize carbon dioxide but obtain energy by oxidizing inorganic compounds; whilst the *Pseudomonodaceae* are heterotrophic, having lost the ability to utilize carbon dioxide and radiant energy. The *Chlorobacteriaceae* are taken to represent a second line of evolution from the common stock.

This argument hinges entirely on the assumption that chlorophyll was only evolved once. Studies on the mechanism of carbon dioxide fixation in autotrophic bacteria are urgently required, for should it prove that the mechanism is the same as that in photosynthetic organisms this would provide additional support to the above scheme. It is also important to investigate the distribution of the green plant carbon dioxide fixation mechanism, for should these reactions be more widespread than at present appears to be the case then the hypothesis would be weakened.

To conclude, I think it can be argued that in some instances physiological characters can be of use in the development of a phylogenetic system of classification, but so far the use is limited. The question of the relationship, if any, of the organisms already discussed, which do appear to fit into one unit, with the remainder of the Eubacteria, cannot be decided at present. We cannot decide because we have not sufficient evidence. This is a depressing admission, but it must not be concluded that a system of classification of bacteria which at the same time indicates phylogenetic relationships is impossible to attain. The only conclusion we can draw is that the present approach is inadequate. I have no doubt that, in time, characters will be discovered or rediscovered which, when their significance is appreciated, will enable us to build such a system.

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Methods for Determining the Biochemical Activities of Micro-organisms as applied to Classification

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We all know what we mean by biochemical methods, although it is unlikely that we all mean the same. We may differ in the biochemical criteria we use for classification, and also in the techniques we prefer for employing them.

The main methods in general use can be divided as in Table 1. In addition to providing information about the synthetic abilities of micro-organisms, nutritional studies enable us to prepare chemically-defined media. Most of

Table 1. *Biochemical methods used in classification*

1	Minimal growth requirements	Nutritional studies
2	Substrate utilization for growth and energy source	Including metabolic pathways to some extent
3	End products of metabolism	From complex media; single carbohydrates or any other compound
4	Identification of characteristic compounds including enzymes	Gums, capsules, toxins, storage products, etc.

the tests are concerned with metabolic breakdown (groups 2 and 3), and here the varied and often extreme metabolic behaviour shown by micro-organisms makes it possible to describe them by the substrates they metabolize and the compounds they produce. Many micro-organisms have characteristic and biologically unusual end products of metabolism: for example, *Thiobacillus thio-oxidans* which produces up to N/10 sulphuric acid; so that we often encounter organisms, the biochemical equivalent in curious development of the sabre-toothed tigers.

Using simple and established methods for each of these groups, we could very rapidly find out this information about the habits of *Staphylococcus aureus*. For growth and energy it requires organic carbon and CO₂. It will not grow on an ammonium salt medium, and requires the addition of a source of growth factors to a protein hydrolysate. Further study will show that it requires a certain number of amino acids and growth factors. We find that it ferments a number of carbohydrates with the production of acid but no gas; it produces acetoin from glucose, and ammonia but not indole from peptone; reduces nitrates to nitrites; liquefies gelatin; and produces a coagulase, haemolysins and an enterotoxin.

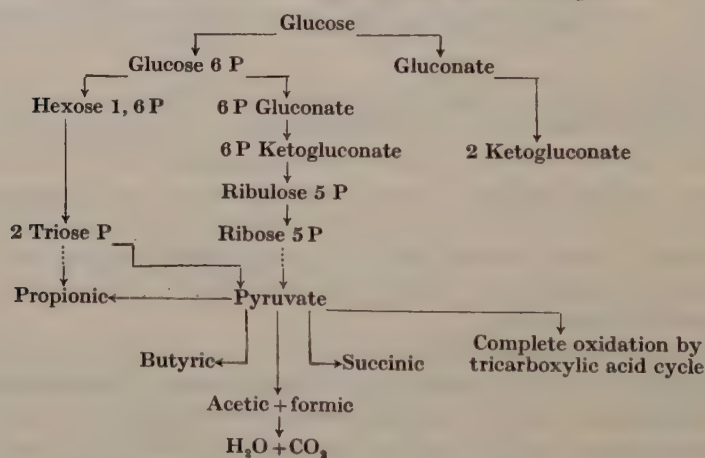
From the point of view of classification in the abstract, it is irrelevant how difficult it is to determine any property about a living organism if it can be

done at all, and if it can be repeated. With micro-organisms, it is a distinct advantage to be able to study large numbers of strains, to be sure that the character is not confined to a few only. This does not mean that observations should be limited to growing cultures in test tubes. Techniques may involve microtests, plate cultures, washed suspensions or extracts, but the ideal to be aimed at is an elegant simplicity of technique.

These are the methods I am concerned with here—methods in constant use by microbiologists whereby some of the biochemical characteristics of a large number of strains can be studied, and I shall make a few tentative suggestions for possible developments. Most of my examples will be drawn from the heterotrophic group of bacteria, because they have been more intensively studied than any other group of micro-organisms.

Many of the tests we use have been borrowed from the diagnostic laboratory. The main requirements for diagnosis are a limited number of speedy and reliable tests which will pick out the suspected organisms. In applying biochemical tests to classification we also want to find out as much as we can about the biochemical behaviour of the organisms which would have escaped through the diagnostic sieve and to assess the significance of our findings. Not all our tests are of equal importance. In devising a rational scheme of classification we should like to assign a hierarchy of subdivisions according to the relative importance of the different characters, but this is not easy to do.

Table 2. *Breakdown of glucose and other carbon compounds*



In the breakdown of glucose and other carbon compounds (Table 2) many fungi and a few bacteria such as the pseudomonads may metabolize glucose by direct oxidation, other fungi and bacteria use the oxidative monophosphate pathway, and the lactobacilli an anaerobic variation of it. Algae and some bacteria use the glycolytic pathway, with or without a final stage of oxidation by the tricarboxylic acid cycle or otherwise. These divisions by metabolic pathways are more fundamental than divisions on the basis of whether two disaccharides

such as maltose and sucrose are both metabolized by two organisms. More than one pathway may be present in one organism; and if so, various factors will determine how much glucose is metabolized in each way.

Have we any tests which will show these differences? Barron & Friedmann (1941) showed that glucose utilization by *Pseudomonas aeruginosa* was not inhibited by fluoride, suggesting that it was not metabolized exclusively by the glycolytic pathway. This could be applied as a test to detect organisms not dependent entirely on the glycolytic route of glucose breakdown. Hugh & Leifson (1953) suggest a simple method for distinguishing bacteria which primarily ferment glucose from those which metabolize it by oxidation. They incubate two tubes of medium so that one will show anaerobic metabolism and the other aerobic metabolism. From their results they divide the organisms they have studied into three groups (Table 3): (1) organisms which neither

Table 3. *Use of glucose metabolism in classification (after Hugh & Leifson)*

Species	Glucose		Metabolism
	Aerobic	Anaerobic	
<i>Alkaligenes faecalis</i>	—	—	(1) Non-oxidizer, non-fermenter
<i>Pseudomonas aeruginosa</i>	A	—	(2) Oxidizers, non-fermenters
<i>Bacterium anitratum</i>	A	—	
<i>Shigella dysenteriae</i>	A	A	(3a) Fermenters (anaerogenic)
<i>Vibrio cholerae</i>	A	A	
<i>Salmonella enteritidis</i>	AG	AG	(3b) Fermenters (aerogenic)
<i>Escherichia coli</i>	AG	AG	
<i>Klebsiella aerogenes</i>	AG	AG	

oxidize nor ferment glucose; (2) organisms which oxidize but do not ferment; (3) anaerogenic and aerogenic fermenting organisms. Again, the ability to attack gluconate has been shown to be a useful classificatory character. Haynes (1951), in studying the characteristically oxidative pseudomonads, tested a large number of strains for their ability to produce 2-ketogluconate from potassium gluconate. De Ley (1953) has shown that several bacterial species metabolize gluconate; and Shaw (personal communication) found it a useful character in subdividing the Enterobacteriaceae, although the route and end products were not known.

The end products of metabolism of carbohydrate are usually recorded as acid, or acid and gas. For some purposes, and in considering closely related organisms, acid and gas may be a sufficient statement; but it is often important to know 'what acid and what gas'. The propionibacteria, the anaerobic butyric bacteria, and many other groups of bacteria and fungi, have well-known and characteristic end products of fermentation. In general, apart from a few special cases such as the production of acetoin from glucose, there are no simple tests for end products, although for more detailed study new methods are available such as the elegant gas liquid partition chromatography method of James & Martin (1952) for fatty acids.

A simple and general test for the ability to use the simple organic acids is

based on the development of an alkaline reaction when the organisms are grown on a simple medium containing the sodium salts of the organic acids. Kauffmann (1951) used this method for testing the activity of the Enterobacteriaceae towards citrate, tartrate, etc.; and Leifson (1933) used malonate in this way to differentiate *Escherichia coli* from *Klebsiella aerogenes*. It is a method of very wide application.

It is unlikely that these simple tests, used alone, will reveal fundamental biochemical differences; they are no substitute for the intensive biochemical study of enzyme systems. It does not follow, for example, that if an organism does not grow on a simple citrate medium, it is completely unable to metabolize citrate. *Escherichia coli* is said to be citrate-negative, it produces no visible growth on a citrate medium; yet most strains will grow if supplied with a little glutamic acid as well, and cell-free extracts from washed suspensions have been shown to metabolize citrate and to contain the citric acid cycle enzymes. The difference between citrate-negative and citrate-positive organisms may be one of permeability, but it is none the less useful in classification.

Compared with carbohydrates, the activity of micro-organisms towards nitrogen compounds has been neglected for classification. The usual tests include only the digestion of gelatin and, as far as amino acids are concerned, the production of indole from tryptophan and H_2S from sulphur-containing amino acids in complex organic media. The last has been confused by the habit of some authors of adding inorganic sulphur compounds such as sulphite or thiosulphate to their media.

But micro-organisms can attack nitrogen compounds in almost every conceivable way, and we might look here for some new tests. For example, we could exploit the differences in amino acid metabolism. Gale (1946) found that the amino acid decarboxylases of the species he examined had a definite and limited distribution. Sharpe (1949) used a test based on tyrosine decarboxylase to separate *Streptococcus lactis* and *S. faecalis*. Møller (1954) has recently surveyed several hundred strains of species of the Enterobacteriaceae, and found it possible to distinguish groups by their decarboxylases. King (1953) found that *Proteus vulgaris* produced decarboxylases for valine and leucine; this fitted with Proom & Woiwod's (1951) amine test for *Proteus* species.

Table 4. Use of amino acid decarboxylases in classification

	Bacterial amino acid decarboxylases								
	Hist.	Arg.	Lys.	Orn.	Tyr.	Glut. A.	Asp.	Leuc.	Val.
<i>E. coli</i>	+	+	+	+	+	+	+	-	-
<i>K. aerogenes</i>	+	-	+	+	-	-	-	-	-
<i>P. vulgaris</i>	-	-	-	+	-	+	-	+	+
<i>S. faecalis</i>	-	-	-	-	+	-	-	-	-
<i>C. welchii</i>	+	-	-	-	-	+	+	-	-

Hist. = histidine, Arg. = arginine, Lys. = lysine, Orn. = ornithine, Tyr. = tyrosine, Glut. A. = glutamic acid, Asp. = aspartic acid, Leuc. = leucine, Val. = valine.

Stumpf & Green (1944) described an L-amino acid oxidase of *Proteus vulgaris* which could attack most of the natural amino acids by oxidative deamination. Clarke & Shaw (1954) have tested for this enzyme among the Enterobacteriaceae with phenylalanine as the substrate, using both growing cultures and a very simple test with washed suspension. Among several hundred strains of various species shown in Table 5 they found that this enzyme and also leucine decarboxylase were found at an appreciable level only among *Proteus* species and the Providence group. The L-amino acid oxidase of *Proteus* is a case of excessive production of an enzyme by a group of bacteria. It can be detected in some other species at a very much lower level. These two sets of tests are more precise than the older description of *Proteus* as 'producing amines', and 'producing ammonia from peptone'.

Table 5. *Division of Enterobacteriaceae by the phenyl alanine test*

Organism	Oxidative deamination of phenyl alanine	
	No. of strains	
	+	—
<i>Proteus</i> spp.	62	0
Providence group	125	0
Arizona group	0	54
Bethesda-Ballerup group	0	9
<i>E. coli</i> I	0	43
<i>Klebsiella</i> spp.	0	68
<i>Shigella</i> spp.	0	20
<i>Salmonella</i> spp.	0	211

I mention these studies because the methods are very simple tests for single enzymes, and can readily be fitted into a series. A few tests for single enzymes are already widely used, such as urea splitting by urease, or indole production from tryptophan, although the latter is usually carried out on complex media—peptone or broth. The advantages of using a defined substrate and/or chemically defined media are obvious. There is considerable scope for developing many more methods both for classification and also for diagnostic tests on these lines.

Most of the methods to which I have referred are empirical. I am suggesting that having applied to classification our knowledge of energy sources, synthetic abilities and metabolic pathways, we subsequently utilize any character which varies in a consistent way, even if its metabolic significance is not known. So that on the one hand we want to develop tests which are related to the more fundamental differences, and on the other to exploit any enzyme system of uneven distribution for classification at the finer subdivisions.

The possession of a common enzyme is not by itself evidence for close relationship of organisms. It may only mean that the organisms live in the same kind of environment and metabolize the same substrates. Studies of enzyme distribution must be evaluated against the other characters of the organism including morphology, serology and any toxin production. It would

be artificial to rank these characters in a rigid order of importance for all groups. It can be argued that fundamentally they are all biochemical. The toxins whose modes of action have been elucidated have been shown to be enzymes and there is no reason to suppose that collagenase and hyaluronidase are any less or more important than urease or glutamic acid decarboxylase. Even the demonstration of morphological features depends on the presence of the necessary chemical grouping to react with the appropriate stains.

What sort of results can we expect to get? If we carry out a series of quantitative studies on any particular enzyme system on a number of strains of the same species, we will find a very large variation in activity. Even if we take single cells, we will find that without any artificial aids to mutation they may throw off variants with more, less or none of the activity we are investigating. If we start with an organism possessing a certain number of biochemical characters by means of which *inter alia* we define it, then it may lose a certain number of these characters, and we will still describe it as a variant of the original species. Where we draw the line is a matter of taste, and the more organisms we study in detail, the more blurred become the lines between species and between genera. The biochemical plasticity of micro-organisms, enabling them to multiply in more than one environment, results in this accumulation of surplus enzyme potential, some of which is readily lost. We may therefore find that we can best express some of the biochemical characters on a statistical basis, or more simply describe characters as invariably positive, invariably negative, usually so, or variable.

Finally, although it is a difficult subject for general discussion of this kind, I must refer briefly to experimental details. Many differences of opinion about biochemical reactions, for example whether or not *Tetrahymena* ferments certain sugars, have been due to differences, often slight, in the methods used. Over a limited range of organisms it is often possible to devise a standard method, and when this can be done it is more important to have an agreed method than an optimum one. In general, one can say that no description of biochemical characters is complete unless the method is indicated. The *Bergey Manual* (1948), for example, gives no indication of the validity of the characters they list and some of them mean very little. 'Nitrates reduced' or 'nitrates not reduced' is a familiar expression, but as Kluyver (1953) pointed out, the absence of a positive reaction for nitrite after an organism has been grown for a few days on a broth or agar containing potassium nitrate has more than one interpretation. It may mean that no nitrate has been reduced to nitrite or that reduction has proceeded beyond the nitrite stage. In this case we can easily arrange to get more precise information about what is happening to the nitrate, and the more unequivocal we are able to make these biochemical tests the more use they will be for classification.

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Bacterial Toxins and Classification

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To be widely useful in classification, a character should be reasonably easy to demonstrate, it should be consistent, and its incidence should be known over a wide range of organisms. On all three points the production of a particular soluble bacterial antigen is hardly satisfactory as a diagnostic criterion, as it is inconstant in a particular species, or even in a particular strain, its demonstration often requires complex immunological procedures, and far too little is known about its distribution. I have been asked to speak about bacterial toxins, but prefer to consider soluble bacterial antigens in general; the killing power of some bacterial antigens is, of course, practically important, but since it is dependent on concentration as well as on existence, it is hardly a true discriminant.

The degree to which the soluble antigens of bacteria have been examined depends very largely on their real or imagined importance in human or veterinary medicine, and on the personal interests and opportunities of those who have examined them. So it is not very surprising that the soluble antigens that have received the greatest attention are the bacterial toxins, particularly those of the genera *Corynebacterium*, *Staphylococcus*, *Streptococcus*, *Shigella* and *Clostridium*, while the soluble antigens of non-pathogenic organisms have hardly been seriously examined at all.

Our ignorance of the distribution of soluble bacterial antigens is matched by our ignorance of the mode of action of the few we know something about; for apart from the lecithinases, collagenases and hyaluronidases, whose action is to some extent understood, we have as a rule little idea of the fundamental actions of bacterial toxins. Thus *Clostridium welchii* produces eight or nine lethal substances, only two of which (the lecithinase α and the collagenase κ) have known modes of action; the action of the rest is unknown, and there seems no reason to suppose that it is the same for any two of them. So that any attempt to use bacterial antigens in classification is limited by our ignorance, as well as by the regrettable habit bacteria have of ceasing to produce antigens that are regarded as characteristic of them, or of producing them only in circumstances that are very complex and difficult to repeat. Moreover, concentration of bacterial filtrates may show that traces of active material are present, though they cannot be demonstrated in the unconcentrated material.

As far as I can see, bacterial toxins are chiefly of value, and of very considerable value, too, in examining the relationships within a genus, and deciding whether a particular classification is useful and convenient or not. Thus *Clostridium welchii* produces a set of soluble substances, mainly dis-

criminated by immunological means, by the use of which it is possible to divide the strains of this species into six groups. All the groups possess certain soluble antigens in common, and it is obviously convenient that the antigen should be called by the same name whichever group it occurs in. For this reason I prefer to divide *C. welchii* into six types, so that whether I am speaking of *C. welchii* type B, or *C. welchii* type C, or *C. welchii* type F, I can speak of the β toxin of *C. welchii* without ambiguity. Prévot's classification, by which *C. welchii* is divided into two species; *Welchia perfringens* with its varieties *egens*, *zoodysenteriae* and *vitulitoxicus*, and *W. agni* with its varieties *paludis*, *wilsdoni* and *hominitoxicus* seems to me to be inconvenient in this respect—what are we to call the toxins?—and to have little backing from other methods of classification, as the organisms are far too much alike, colonially and biochemically, to justify their separation into species.

The lethal toxins of *Clostridium oedematiens* and *C. gigas* are immunologically indistinguishable; this supports the other colonial and biochemical characters by which they are classified together as types of *C. oedematiens*; their distinctness as types is emphasized not only by differences in size and biochemical activity, but also by the fact that their lecithinases (γ and β) are immunologically distinct. *C. oedematiens* type C from the Dutch East Indies does not certainly produce any identifiable soluble antigens, and its relationship to *C. oedematiens* has been demonstrated by other means. *C. haemolyticum* is closely related colonially and biochemically to *C. oedematiens*, but is usually differentiated from it because its lethal toxin is distinct from that of *C. oedematiens* types A and B, and the pathological picture it produces is different. Immunological investigation of its toxin shows, however, that its lethal toxin is antigenically equivalent to the β toxin of *C. oedematiens* type B, but that no α toxin is present. It seems convenient therefore to call *C. haemolyticum*, *C. oedematiens* type D, and to differentiate the types by their production of $\alpha\gamma$ (A), $\alpha\beta$ (B), no toxin (C) or β (D).

Similar arguments of convenience apply to *Corynebacterium diphtheriae* and *C. ulcerans*. Some strains of *C. ulcerans* produce diphtheria toxin, and it seems reasonable therefore to call the *ulcerans* strains *C. diphtheriae* var. *ulcerans*, rather than to separate them as a species.

A useful case to consider here is *Clostridium botulinum*. As far as I know, the toxins of all toxigenic strains of *C. botulinum* act in the same way, or at any rate on the same structures, but the only ones that show any antigenic relationship are those of types C and D. Now it has been suggested that *C. botulinum* should be divided into *C. parabotulinum* (proteolytic) and *C. botulinum* (non-proteolytic). Luckily this brings types C and D into the same species (*C. botulinum*), which seems to me very much more convenient than separating them, and to be a case in which antigenic structure does not oppose conclusions based on biochemical activity.

One more use of the minor antigens of a species is to identify degraded strains; *Clostridium welchii* strains occasionally lose their capacity to produce their main lethal toxins, and one may have to fall back on the others. Thus a *C. welchii* strain producing δ and κ is very likely to belong to type C, though

it does not produce β , while one producing β , λ and μ is very likely to be a type B, though it does not produce ϵ .

Can we apply our knowledge of soluble bacterial antigens to larger groups than species? Not, I think, even in the most tentative way. Thus *Clostridium oedematiens*, *C. welchii*, *C. bifermentans*, all produce lecithinases that act in the same way, by splitting unsaturated lecithin into stearyl-oleylglyceride and phosphorylcholine. It is true that there are slight differences in small matters, but the striking fact is the equivalence of the main activity. Notwithstanding this, the *C. oedematiens* lecithinases differ antigenically from one another and from the lecithinase of *C. welchii*. *C. bifermentans* lecithinase, on the other hand, though it differs in some points from the lecithinase of *C. welchii*, has some antigenic relationship with it. But this antigenic relationship between their lecithinases can hardly be taken as evidence that *C. bifermentans* and *C. welchii* are very closely related, for they differ very markedly in their biochemical activities, while *C. welchii* and *C. oedematiens*, whose lecithinases have little, if anything, in common, appear in other respects to be fairly closely related. Perhaps the worst case for the use of antigenic differences is *C. oedematiens* itself, for two of its types, A and B, though they share the lethal antigen α , produce antigenically distinct lecithinases γ and β . This does not encourage the use of antigens having the same enzymic activity to define a group, and though *C. welchii*, *C. oedematiens* and *C. bifermentans* are usually classed together in the genus *Clostridium* for what appear to me to be valid reasons, I cannot imagine that removal of *Bacillus cereus* and *B. mycoides* to the genus *Clostridium* because they produced a lecithinase would be very popular, or even justifiable, even though the lecithinases of *B. cereus* and *B. mycoides* are antigenically related.

The collagenases of *Clostridium welchii* and *C. histolyticum* are antigenically distinct, though they probably act in a similar way; *C. septicum* and *C. welchii* hyaluronidase are distinct; but in both cases the differentiation between the species has been made by other means.

I do not think that the oxygen-labile lysins give much information. They all have certain properties in common, and there is some evidence that they are antigenically related, but a consideration of the species known to produce them—*Clostridium welchii*, *C. septicum*, *C. histolyticum*, *C. tetani*, streptococci and pneumococci—hardly inspires much confidence in their usefulness for classification.

In the present state of our knowledge, soluble bacterial antigens can be used, it seems to me, only to divide species, devised on other criteria, into types or similar smaller groups. As far as I know, no example exists of the same soluble antigen being shared by two obviously unrelated bacteria, but even if it did occur, we could get over it by insisting that in classification all characters ought, in theory at least, to be taken into account, and not only those that seemed important to the investigator, possibly only because he was working on them. Obviously far more work is necessary to make soluble antigens more than adjuncts to the usual means of classification.

Finally, I should like to emphasize the value of the minor soluble antigens.

They can often be worked on with very little apparatus, and no animals, and I feel that they would well repay investigation, if only to clear up discrepancies in testing for the 'major' ones.

DISCUSSION

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It would have a most salutary effect if the good example set by Oakley's cautious assessment of the taxonomic significance of soluble antigens (toxins) were followed by workers concerned with non-soluble bacterial antigens.

Nutritional Characters

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The purpose of this contribution is to suggest that at least certain of the nutritional requirements of micro-organisms are sufficiently stable and characteristic as to be of use in classification. This may at first seem surprising in view of the relative ease with which nutritionally exacting mutants can be obtained by the use of mutagenic agents. But there is evidence that under natural conditions and in normal laboratory maintenance on complex media (i.e. under conditions when more exacting mutants would not be selected) the nutritional requirements of micro-organisms defined on other grounds are often very characteristic. Although the easily testable nutritional requirements are perhaps not very numerous, yet sufficient variety exists to make the examination of nutritional requirements a useful method in classification. This conclusion may be illustrated by four examples.

(1) *m*-Inositol. This compound was one of the earliest growth factors to be isolated and chemically identified when it was found to be a growth factor required by certain yeasts (Eastcott, 1928). *m*-Inositol was subsequently found to be a nutrient required by other Eumycetes (others of which synthesize it). But there appears to be no authenticated report of *m*-inositol being a required nutrient among the Schizomycetes, although a few (all examined) have been shown to synthesize it (e.g. Thompson, 1942). Without further documentation it may be said summarily that *m*-inositol bears the character of an essential metabolite, i.e. a substance required in the growth of many different organisms and which, when it cannot be synthesized endogenously, becomes a nutrient requirement and must be supplied preformed. It is very surprising that no schizomycete which requires *m*-inositol as a nutrient has yet been reported, while this requirement has been observed fairly frequently among the Eumycetes. It might be argued that for some reason unknown a failure of inositol biosynthesis in a schizomycete is lethal and cannot be circumvented by the utilization of externally supplied *m*-inositol as it can in certain Eumycetes. When inositol-dependent mutants of *Aerobacter aerogenes*, which according to Thompson (1942) synthesizes *m*-inositol, were sought in screening experiments after treatment with mutagenic agents, none was found (Dr G. Pontecorvo; private communication). This supports the significance of the absence of reports of schizomycetes with nutritional requirements for *m*-inositol. There is thus some evidence that Eumycetes and Schizomycetes are different in their relation to *m*-inositol; a deficiency in biosynthesis of *m*-inositol can be circumvented by an external supply of it in Eumycetes, whereas such a deficiency either cannot arise in Schizomycetes without being lethal and is not remedied by externally supplied *m*-inositol, or *m*-inositol is

not a general essential metabolite for these forms. In any case the distinction is interesting; it might reflect some phylogenetic difference. Whether the difference in relation to *m*-inositol can be correlated with some other character which separates these great subdivisions of the Thallophyta remains to be studied.

(2) In the genus *Haemophilus* are the classical organisms which exhibit nutritional requirements for one or both of the growth factors originally named, respectively, the V factor (now known to be one or other of the pyridine nucleotide co-enzymes or certain precursors) and X factor (certain porphins) as minimal nutrients. The designation of nutritional requirements as characteristic of these organisms is of surprisingly old standing (Kristensen, 1922; Fildes, 1923) and is used in *Bergey's Manual* (6th ed., 1948). Incidentally, an organism which was classified as *H. duplex* in the previous (5th) edition of *Bergey's Manual* (1939) was transferred to *Moraxella* as the species *M. lacunata* in the 6th (1948) edition largely on the evidence and arguments of Lwoff (1939) and Audureau (1940) who adduced nutritional requirements as part of the characterization in *Moraxella*. The removal of the moraxellas from the genus *Haemophilus* renders the nutritional characterization of this genus much more homogeneous. This process is carried still further by the study of Proom (1955) on the nutrition of strains of pertussis, parapertussis and bronchiseptica which 'supports the already impressive evidence that these groups should be classified separately'. Proom found that all the strains examined had a nutritional requirement for nicotinic acid and no other vitamin, and had other nutritional and physiological similarities. In all, the nutritional findings offer strong support to the proposal of López (1952) of a new genus *Bordetella* to include *Bordetella pertussis*, *parapertussis* and *bronchiseptica*.

(3) Athiorhodaceae. Van Niel (1944) made a very thorough study of the ecology, morphology and physiology of the photosynthesizing bacteria belonging to the family Athiorhodaceae and eventually recognized six well-defined species distributed in two genera. Subsequently, Hutner (1946, 1950) examined the nutrient requirements of van Niel's strains with respect to biotin, nicotinic acid, *p*-aminobenzoic acid and thiamine. Each van Niel species had a characteristic nutritional pattern, the intraspecific variation being remarkably small. For example, *Rhodospirillum rubrum* usually required biotin only, *Rhodopseudomonas spheroides* required biotin + thiamine + nicotinic acid, *R. palustris* required *p*-aminobenzoic acid (9 of 10 strains examined). The correlation between the other characters used to define species and the specific nutrient patterns is so close that strains could be assigned to species on nutritional pattern alone with little likelihood of error.

(4) The genus *Bacillus*. The differentiation of mesophilic species in this genus has been well worked out by Smith, Gordon & Clark (1946, 1952). Their earlier (1946) classification was adopted in *Bergey's Manual* (6th ed., 1948). They divided the genus into three groups based on the morphology of the spores and sporangia. The subdivision into species and varieties was made upon physiological characteristics. In the 1946 monograph of Smith *et al.* there were fifteen species and seven varieties. This contrasted with the

150 or so differently named cultures which they examined and the twenty-six pages in double column of unallocated and insufficiently described strains recorded in the appendix in *Bergey's Manual* (1948). Knight & Proom (1950) collected about 300 fresh isolates of mesophilic *Bacillus* species from soil and classified them according to Smith *et al.* (1946). A good number of named laboratory strains was also collected and their characters checked in the same way (not all the names were substantiated). From this collection of fresh isolates and laboratory strains about 200 were examined for their nutritional requirements with regard to ammonia or amino acids and various growth factors (biotin, nicotinic acid, pantothenic acid, thiamine). Since the isolation of the strains from soil had taken place in nutritionally rich media it was reasonable to assume that no selection for simpler nutrient requirements had taken place and that the nutritional patterns of the fresh isolates did represent those of the organisms living in the soil. It was found, in general, that the nutritional patterns of new isolates of a species as defined by Smith *et al.* (1946) and the corresponding laboratory strains agreed very well. Thus the nutritional characters had survived what was in many cases long-continued laboratory cultivation. This already indicated a certain stability of the nutritional patterns. What was perhaps more surprising was the finding that there was a close correlation between the patterns of morphological and physiological characters used by Smith, *et al.* (1946) to define their species, and the corresponding nutritional patterns. Within the collections of strains of the same species there was a surprising uniformity of nutritional pattern. The new species *Bacillus pantothenicus* described by Proom & Knight (1950) was clearly characterized by its requirement for pantothenic acid—unique among the mesophilic *Bacillus* spp.—as well as by its morphology and other physiological characters. These specific nutritional patterns thus appeared to be highly characteristic of these species of *Bacillus* defined on quite other grounds, just as Hutner found with the Athiorhodaceae. Stanier (1953), in discussing this kind of finding with the Athiorhodaceae, said:

Since we know that a vitamin requirement can be brought about by a single gene-mutation, there was certainly no *a priori* reason for expecting that in a group where dependency on an exogenous vitamin has become the rule, the pattern of [nutritional] dependency should be so rigidly fixed for each species, particularly since the general ecology of the group, governed by its unique photosynthetic properties, is both narrow and uniform. One is forced to conclude that the special constellation of vitamin requirements which characterizes each species must possess great adaptive value, either direct or indirect, although the reason for this remains wholly obscure.

This outlook applies equally well to the nutrient requirements of the species of *Bacillus*. As Stanier further pointed out, even highly mutable single-gene characters '...may often be perfectly good taxonomic ones, if they possess adaptive value in a natural environment and are consequently maintained by selection'. In the case of *Bacillus* nutrition the comparison between fresh isolates and old laboratory strains did not suggest that the nutritional patterns were highly variable. The patterns of nutritional requirement shown, for

example among the *Bacillus* species, reveal biosynthetic deficiencies. It should not be assumed that these deficiencies are due only to single-step biosynthetic defects or single-gene mutations or that the character could only be maintained by selection (see Stanier above). During the course of time multiple deficiencies in the same biosynthetic pathway may have accumulated which, alone, would render these nutritional patterns much more stable.

With the examples of *Haemophilus*, the Athiorhodaceae, *Bordetella* and *Bacillus* before us (and other examples could be adduced) it appears that there is now ample evidence to suggest that the nutritional patterns of microbial species may be among the properties always well worth considering as characters to use in classification.

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Host-parasite Relationships

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There were taxonomists before there were biochemists and serologists, cytologists and geneticists, and modern taxonomic practice as applied to both macro- and micro-organisms shows abundant evidence of the naturalist's traditional approach. Both before and after the application of diverse special techniques to taxonomy, however, systematists have frequently felt it necessary to employ criteria which would allow them to summate characteristics which could be defined only by workers in other disciplines. Among such criteria the relationship of the parasite to the host takes pride of place for pathogenic micro-organisms and it is to aspects of this relationship that I wish to draw attention. The host-parasite relationship could be discussed comprehensively only on a symposium scale, for in addition to the species concept, the genetics and physiology of both host and parasite are among the many major questions involved and I must, therefore, confine myself to illustrating by examples chosen mainly from the fungi two aspects of the problem: the taxonomic use of what may be termed the spatial relation of the parasite to the host and of the host specialization shown by the parasite.

The spatial relationship

The spatial relationship of the parasite to the host, that is, the localization of the pathogen to certain organs or tissues, has been used as a criterion for differentiating generic, specific, and intra-specific taxa. Some of the most familiar examples of the use of these, what Ciferri (1952) called 'ecological', characteristics are provided by the Fungi Imperfecti among which the genera *Phoma* and *Phyllosticta* are differentiated by the occurrence of the former on the stems and the latter on the leaves of the host plants. A similar, if not quite so clear-cut distinction is made between the allied pair of genera *Diplodina* (usually on stems, rarely on leaves) and *Aschochyta* (usually on leaves, less frequently on stems) in which the spores, produced as in *Phoma* and *Phyllosticta* in small flask-shaped structures (pycnidia) immersed in the host tissue, are 2-celled instead of 1-celled as in the first pair of genera. Another example at the generic level is provided by the Dermatophytes in which *Epidermophyton* is distinguished from *Microsporum* and *Trichophyton* by the inability to attack hair. On the other hand, the localized sporulation shown by the hair-invading ringworm fungi on the surface of (in the 'ectothrix' type), or inside (the 'endothrix' type), the infected hair has also been advocated as a generic or infra-generic criterion. The smut fungi (Ustilaginales), an ubiquitous order of plant pathogens, provide instances of the differentiation of species and varieties by

similar criteria. For example, *Ustilago lygei* was differentiated from the morphologically similar stem smut of grasses, *U. hypodytes*, by reason of attacking the inflorescence and not the culm while the same differential character was used to distinguish *U. tritici* var. *foliicola* as a leaf-invading variety of the loose smut of wheat (*U. tritici*) which is normally confined to the inflorescence.

The physiologic relationship

Although host specialization has been much more extensively employed as a taxonomic criterion than has localization of the parasite on the host it has rarely been used at a generic or supra-generic level. Among the better known examples, the separation by Bergey *et al.* of plant pathogenic bacteria from similar coliform organisms as the tribe *Erwiniae* comprising the genera *Erwinia* (flagella peritrichous) and *Phytomonas* (flagella polar or absent) may be recalled. For specific and infra-specific taxa host specialization (or alleged specialization) has been exceedingly popular as any check-list of plant pathogenic fungi suggests. Up to 50 % or more of the specific epithets are found to be derived from host names.

The reality of biological races is indisputable and their existence has for long tried the patience and exercised the ingenuity of both taxonomists and plant pathologists. It was among the rust fungi (Uredinales), a group of obligate plant parasites, that biological races were first recognized about 60 years ago, and since then the smuts and many other groups of fungi have been shown to exhibit the same phenomenon. Among the smuts, morphologically similar forms cause similar diseases in wheat and barley but the fungus from wheat will not infect barley and vice versa. This host specificity led to the erection of the two species, *Ustilago tritici*, the cause of loose smut of wheat, and *U. nuda*, the cause of loose smut of barley. Sometimes small biometrical differences can be detected between such forms, but usually any morphological differences are too slight to have any practical taxonomic value and the taxonomic treatment is frequently modelled on that of the black stem rust, *Puccinia graminis*. This rust exhibits a series of races specialized for the parasitism of a range of cereals and grasses and it has been customary to treat these races as 'formae speciales' designated by Latin epithets (e.g. *Puccinia graminis* f.sp. *tritici*) to give names which have been used by many cereal pathologists and others as trinomials (e.g. *P. graminis tritici*). By using a series of carefully selected pure bred varieties of the host it is possible to demonstrate within one forma specialis the existence of large numbers of subraces (some 300 for *P. graminis tritici*) for the nomenclatural treatment of which the International Code of Botanical Nomenclature provides no guidance. A knowledge of such races and exchange of information on their geographical distribution is of great practical importance to plant pathologists who, therefore, agreed among themselves on an unofficial registration of these races at Prof. Stakman's laboratory at the University of Minnesota which acts as an international clearing house and allocates numbers by which the different races are distinguished.

There is a further complication. Many rusts are heteroecious. Their life cycles involve pairs of distinct, and frequently taxonomically widely unrelated, hosts. On one host a haploid mycelium undergoes fusion with an element of another haploid mycelium and as a result binucleate aecidiospores are produced. These aecidiospores bring about infection of the alternate host in which a binucleate (dikaryotic) mycelium produces a second type of dikaryotic spore (the uredospore) and finally a third type of spore (the teleutospore) which is diploid. The different formae speciales of *Puccinia graminis* have a common aecidial (gametophytic) host in barberry (*Berberis*). For other morphologically similar races, those of the brown rusts of cereals, for example, several different aecidial hosts belonging to several different families are involved. In such cases it is common practice among uredinologists to accord specific rank to the biological races. The result is a number of morphologically indistinguishable rusts differing in both aecidial and uredospore-teleutospore hosts and much confusion. (For further details the useful review by Dennis, 1952, may be consulted.)

Taxonomic effects

These few examples must suffice as a background for a brief consideration of some of the effects of the use of criteria derived from host-parasite relationships on taxonomic practice.

The spatial relationship is usually employed for reasons of convenience. As Dennis (1946) has pointed out, the traditional allocation of pycnidial fungi to the form genera *Phoma* and *Phyllosticta* according to whether they are collected on stems or leaves has long been recognized as irrational, and its retention is a tribute merely to its convenience and a confession of the necessity of grouping for easy reference the innumerable names that have been applied in the literature to these fungi. The spatial relationship may, however, at times be a constant and reliable feature as is the specificity of *Epidermophyton floccosum* for skin. Such characters are therefore eligible for consideration as taxonomic criteria, but there is a growing weight of opinion among mycologists that it is always best to define the basic taxonomic categories by mycological criteria and during recent years the genus *Epidermophyton* has been acceptably redefined in mycological terms. For the same reason, the names *Ustilago lygei* and *U. tritici* var. *foliicola* have been reduced to synonyms of *U. hypodytes* and *U. tritici*, respectively.

The widespread use of host specialization for differentiating species has presented mycologists with a major dilemma, for in certain groups the easiest way, and sometimes the only way, to identify a fungus is not by a study of the fungus but by identifying the host plant. The use of this method has also been one factor leading to a great multiplication in the number of species. For all Ascomycetes the number of generally accepted species per genus is 8.3 and for 11 of the 14 orders this number varies from 2.3 to 10.5. In the remaining 3 orders, the Laboulbeniales, Erysiphales (powdery mildews), and Taphrinales, all orders in which host specificity has played an important part in speciation,

the number of species per genus are 12.5, 16.7 and 20.8, respectively. For rusts (Uredinales) the number is 36.

There is however a third and perhaps even more important aspect of the impact of the host on speciation. The average mycologist is impressed by the apparently greater frequency among fungi pathogenic for man and animals of fungi which are normally saprophytes. This impression is probably distorted because although several major groups of fungi are composed of obligate parasites of plants the emphasis on host specialization for differentiating species has certainly multiplied plant pathogens at the expense of non-pathogens. For example, most species of *Phoma* that have been described are parasites, but the relation of these forms to one another and to the forms found in the soil and as air-borne spores is obscure. It is almost certain that many of the plant pathogenic species of this genus, reduced to physiologic races and united specifically, could be amalgamated with related saprophytes. The same applies to such a genus as *Fusarium* and it is possible, now that dermatophytes and dermatophyte-like fungi have been recorded from the soil, that under a more natural and rational classification certain ringworm fungi will eventually find a place in the same genera, or even species, as non-pathogenic counterparts.

There is, as already mentioned, a modern tendency to favour the description of genera and species of fungi in mycological terms and whenever possible morphology is given precedence over other considerations. A good example of the application of this principle is provided by G. W. Fischer's *Manual of the North American Smut Fungi*, 1954. In this work morphologically similar smuts, such as *Ustilago tritici* and *U. nuda*, are amalgamated with commendable consistence when related hosts are involved, but the author was unwilling to break with tradition and to unite morphologically similar smuts specialized for hosts belonging to different families. Much work remains to be done before the specialist can be satisfied by an appropriate intra-specific taxonomy and nomenclature for many groups, but there is no reason to suppose that the problem is insoluble. The host-parasite relationship certainly has taxonomic value but for the differentiation of species of fungi I feel that the interests of mycology would be best served by responding to the appeal made a few weeks ago by Snyder & Hansen (1954) that fungi should be classified at the species level 'for what they are, not for what they do or where they occur'.

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The Classification of Viruses

By F. O. HOLMES

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Several revisions and discussions of viral nomenclature have been published in the last few years. In 1953 V. M. Zhdanov proposed a complete set of Linnaean binomials for viruses causing diseases in higher animals and insects (*Opredelitel' Virusov Celoveka i Zhivotnykh*, published by the Academy of Medical Sciences of the U.S.S.R., Moscow, 348 pages). In the same year, V. L. Ryshkov presented an even more extensive system of Linnaean binomials, attempting to show interrelationships between viruses that cause diseases of animals and those that cause diseases of plants (*Sowjetwissenschaft, Naturw. Abt.*, **6**, 151). Both of these authors supported the contention that binomial nomenclature is appropriate for viruses. The same opinion was expressed in 1954 by N. O. Frandsen who reviewed in a thorough and unbiased manner all earlier proposals for viral nomenclature. Frandsen stated that the principle of nomenclature that is chosen ought not to reflect particular systematic concepts of viruses. Instead it ought to provide for the greatest possible continuity of naming, as our knowledge of viral relationships changes and grows with the passage of time. He indicated further that binomial nomenclature is especially well adapted to provide continuity for naming when there is progressive improvement in our knowledge. He felt that Fawcett misinterpreted the nature of binomial nomenclature when he claimed to have combined the advantages of the Johnson-Smith numbering principle with those of binomial nomenclature. Fawcett's 1940 proposal involved dual names of which the first member included the name of the host plant genus in which the virus originally was discovered. Frandsen believed that Thornberry's proposal in 1941 also is not to be regarded as involving the principle of binomial nomenclature, even though it contemplates dual names, because his use of a single genus *Phytovirus* for all phytopathogenic viruses reduces his proposal in reality to a method involving a single rather than a dual designation. Frandsen pointed out that one implication in the use of the principle of binomial nomenclature for phytopathogenic viruses is that variation among viruses centres around a taxonomic unit of a rank that is comparable to that of species among animals and plants. He noted wide recognition of the fact that this implication is essentially justified for viruses, and that it often finds expression in the literature by designation of the entities as species. Moreover, he felt that many investigators have admitted that the experience of the last decade has substantiated the further assumption of a possibility of natural groupings of viruses at a higher level, that of the genus. Frandsen observed that certain groups of viruses having a community of characteristics, but differing more or less among themselves, have come to be recognized.

He concluded that the principal advantage of binomial nomenclature consists in the fact that changes in the surroundings of systematic units can be undertaken according to rules that ensure a comprehensive stability and unity to names. There is the added advantage that each investigator who wants to undertake such changes will be forced to show the precise reason for his proposed changes as opposed to existing conceptions.

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The Classification of Viruses

By C. H. ANDREWES

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Recent discussions have centred rather on the nomenclature than on the classification of viruses. This is due in no small part to the reaction to Dr Holmes's 'Virus Supplement' in *Bergey's Manual*. His names were interesting, challenging ones which provoked controversy; and since they were beginning, if only in a small way, to creep into the literature it was necessary for virologists generally to decide whether to bless or to curse: neutrality was impossible. Holmes's classification of animal viruses, on the other hand, seemed to animal virologists to bear hardly any relation to the facts of life. At this point I shall apologize to our distinguished visitor for being repeatedly very critical of his writings. He and I have argued in public before and he knows, I hope, that however rude I may be, it is with the best will in the world. If he knows his Gilbert and Sullivan he will recall what King Hildebrand said to King Gama in *Princess Ida*: 'We will hang you, never fear, most politely, most politely.' Dr Holmes's is the credit for stimulating great interest in virus taxonomy and nomenclature: his action has also been invaluable—and here I am still trying to be polite—as a glaring example of how not to classify viruses. His family and generic divisions, at any rate amongst animal viruses, are based mainly on the symptoms and pathological lesions produced in infected hosts—barely at all on the properties of the viruses themselves. Now signs and symptoms, together with host and tissue specificity, are amongst the most labile properties of viruses, most easily modified experimentally. Further, very similar symptoms and lesions may be caused by viruses widely differing in all their fundamental properties. Thus Holmes places in one genus, *Molitor*, the utterly dissimilar viruses of warts, fowl-tumours and myxomatosis, having in common the one property of causing striking cell proliferation.

You may have gathered that I do not care for Dr Holmes's classification. I have just seen a copy of that of Zhdanov, which is very similar to Holmes's and is, I feel sure, based on it, as it repeats most of Holmes's most glaring errors and adds new ones of its own. Thus it not only includes the Rous sarcoma, warts and myxoma in one genus (*Tumefaciens*) but throws in, for good measure, the Bittner milk factor, an epithelioma of fish, and molluscum contagiosum. Like Holmes, Zhdanov widely separates dog distemper from so-called ferret distemper, Newcastle disease from fowl plague and cat distemper from cat leucopenia. Nearly all Holmes's names have been changed, presumably because Zhdanov was worried by the absence of an adequately confused synonymy.

Viruses are labile, variable agents and we need to rely on characters which are as stable as possible. At Rio de Janeiro in 1950 the Virus Subcommittee

of the International Nomenclature Committee suggested eight criteria to be used in classifying viruses:

- (i) Morphology and methods of reproduction.
- (ii) Chemical composition and physical properties.
- (iii) Immunological properties.
- (iv) Susceptibility to physical and chemical agents.
- (v) Natural methods of transmission.
- (vi) Host, tissue and cell tropisms.
- (vii) Pathology, including inclusion-body formation.
- (viii) Symptomatology.

The more important characters come early on this list; symptomatology, which Dr Holmes stresses even in his generic descriptions, comes last. Emphasis may be rather different for plant and bacterial viruses and will also vary somewhat as between one group of animal viruses and another.

Following decisions taken at Rio and in subsequent meetings, study groups have been trying to apply these criteria to certain better-known viruses such as those of the pox group and the influenza group; and it is hoped to publish shortly descriptions of viruses of these groups from the point of view of taxonomy. The eight criteria are proving very useful in this attempt.

It was felt at the Rome discussion a year ago that the whole essence of the Linnaean binomial system was that its names were closely linked with taxonomic concepts, from the species level upwards. But some idea of taxonomic relations needs nowadays to precede a Linnaean christening. There was an almost unanimous feeling that some internationally agreed orderly nomenclature for viruses was desirable but that our ideas of virus taxonomy were only embryonic; christening at the embryonic stage of development is usually considered premature. It was suggested, therefore, that interim names, non-Linnaean binomials, should be bestowed on certain groups of viruses; the group-names should carry the suffix 'virus'—for example *Poliovirus*—so that all should know them as something apart from the Linnaean system.

I should like to discuss in a little more detail one of the eight criteria—the immunological one. How important are antigenic differences and similarities as indicators of specific or generic relationships? Three groups of viruses suitably illustrate the point.

The group *Myxovirus* (influenza-like viruses) contains, on the recommendation of the international subcommittee, three group-members or species—influenza A, B and C. *M. influenzae*-A and -B are given the equivalent of specific rank because, first, they are quite distinct antigenically; secondly, they show quite a number of other definite though minor biological differences; these concern morphology, susceptibility to inhibitors, range of variation, pathogenicity for ferrets and mice, epidemiological behaviour. On the other hand, it is recommended that *Poliovirus hominis* contains three serotypes, not three species. This is because there is antigenic overlap amongst them and because the biological differences, which mainly concern ease of adaptation to rodents,

are less striking. But at present to classify the arthropod-borne encephalitis viruses is too difficult. By haemagglutination Casals and Brown place them in two groups, cross-reacting within each group but not from one group to another. Neutralization and complement-fixation tests are more specific but indicate some cross-relations—louping-ill having perhaps a relationship to members of each group. Two genera may be coming into focus here, but what of separation of species? The West Nile, Japanese B and St Louis viruses stand very close together—are they only serotypes? No one would propose to make yellow fever conspecific with any of the others; but most might call the two dengue viruses serotypes rather than species. With Uganda S, Ntaya and so forth, differentiation is mainly serological—what are our guiding principles here?

It is, however, one thing to describe and relate together individual viruses which are closely allied—the equivalents of species and genera—but quite another to classify these into families and higher taxa. Some people hold that viruses may be derived from bits of the host-cell; I do not myself regard this view with great favour but admit that it is an arguable proposition. If it should prove true—that herpes simplex is ultimately derived from human material and the Rous virus from that of the fowl, then any classification of viruses on Linnaean principles makes nonsense. But even if things are otherwise, the agents we call viruses may have come to resemble each other as a result of convergent evolution. It would not surprise me to learn that pox-viruses arose as specialized micro-bacteria and the viruses related to yellow fever as specialized micro-protozoa. The ability of the latter to multiply in either insect or vertebrate would fit with such an idea. If their ultimate origins are so much in doubt, it would be foolish to press on too fast with the designation of Suborders, Families and Tribes.

There is one fundamental question which it would be foolish to ignore. More and more recent work indicates that viruses multiply by some method other than by binary fission. It is suggested in the case of some bacterial viruses that they consist of two parts, an inner core of nucleoprotein and carrying the hereditary characters of the 'phage' and an outer envelope together with a tail consisting of phosphorus-free protein and carrying the apparatus necessary to enable the phage to infect a bacterial cell. The phage sheds this coat when it enters its host cell, the nucleoprotein replicates as such and only at quite a late stage in the developmental cycle does it begin to produce the protein of the outer coat. Such a method of reproduction may appear very different from what we conceive of as happening when a bacterium divides. It may, however, prove to be not wholly different from what is going on inside a cell as a preliminary to the large-scale crude process of binary fission.

The bearing of all this on virus classification is this: if virus multiplication should prove to correspond to the secret processes within a cell, are not viruses really very different from any form of cellular life? In 1939 Dr Holmes classified the viruses as *Vira*, a Kingdom of equal status with animals and plants. In 1948 he had second thoughts and degraded them to an order (*Virales*) of Bacteria. I am wondering if perhaps his first thoughts were not better than his second. I am not trying to answer this question: I hope you will discuss it.

There is a difficulty in separating viruses from bacteria in that the dividing line between them is not as sharp as might appear from what I have just been saying. The rickettsiae are minute obligate intracellular parasites (most of them at any rate), having many properties in common with viruses; yet they are by common usage classed with bacteria. Between them and the bulk of viruses stands the group of large viruses related to that of psittacosis—the Chlamydozoaceae. These are many of them susceptible to sulphonamides, chloromycetin, aureomycin and other antibiotics which do not affect true viruses. They resemble the rickettsiae in many other ways and at least one, that causing heartwater, has been placed by different workers on different sides of the dividing line. The international study group unanimously decided that they should not be reft from their natural relations, the rickettsiae, but should be included with them and outside the true viruses, taking their places in the mouth-filling genera *Miyagawanella* and *Chlamydozoon* of the Chlamydozoaceae. This view was endorsed by the main Virus Subcommittee. If we accept this view and are logical, we shall stop calling these agents viruses. There would then be no excuse for vendors of certain antibiotics to claim that ‘their spectrum of activity includes the viruses’.

The organizers of this discussion have very sensibly placed me, as representing sound common sense, between the two extremists, Drs Holmes and Bawden. Dr Holmes wants to start classifying and naming viruses on Linnaean lines right away. Dr Bawden is almost certain to advise you to have nothing to do with any such proceeding. Frankly, I do not know whether the Linnaean system will ever be generally applied to viruses or not: it well may be, as a matter of convenience. All such systems are a matter of convenience. I am all in favour of adopting a line which leaves it open to those who follow after us to apply this system or not as seems expedient, with the least possible disturbance of accepted usage. But we need to classify viruses *first* and we retard progress by trying to classify before we have the basic facts which alone would justify our attempt. I am sorry to have to prepare a gallows for Dr Holmes, but I am sure he will meet his end bravely.

The Classification of Viruses

By F. C. BAWDEN

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Before considering the criteria to use for classifying plant viruses, it is first necessary to consider for what purpose we are classifying them. This fact applies to whatever is being classified and might seem too self-evident to need saying, except that taxonomists often give the impression that there is only one classification possible in biology; this is one based on what are usually termed 'natural' relationships, by which seems to be meant that the groupings reflect current views on evolution. To speak of classifying plants, for example, almost invariably brings to mind only the classification developed by academic botanists, in which plants are first analysed into the basic units of species and then grouped for phylogenetic reasons into genera, families and orders. There are, though, many other possibilities, and few could be less useful than the botanist's to those who are engaged in the cultivation of plants. The main concern of the farmer or gardener is not whether a plant is graminaceous or cruciferous, but whether it is a useful plant for him to grow or a pernicious weed. To the botanist, couch grass may be a near relative of wheat, and charlock of turnips, but to the farmer one of each pair means a profit and the other a loss, two categories that, to his mind, could not be more unrelated. The farmer needs plants classified in categories that show their agricultural value, the way in which they respond to climate, soil type and manuring, their habits of growth, methods of propagation, and whether they are annuals, biennials or perennials. Such classifications also have the virtue of being factual and permanent.

Similarly, viruses can be classified for the farmer or field pathologist quite objectively, by using such criteria as the susceptibility of specific plants to infection, the kind of symptom evoked, the method of spread and, if spread by arthropods, by the species of the vector. To expect such criteria to reflect any phylogenetic relationships between the viruses placed in any one category, however, would be as unrealistic as to expect the farmer's classification of plants to coincide with the botanist's. I imagine that to-day we are less concerned with utilitarian groupings of viruses than with classifications that will be acceptable to the taxonomists of microbiology. Let it be said at once that such classifications are not only more difficult to make, but they must be more equivocal and they also lack any agreed criteria. Indeed, it is even doubtful whether viruses are suitable objects for attempting to arrange in the kinds of groups that are used to classify organisms; the determination of such groups depends on sexual reproduction and phylogeny, criteria that could hardly be less appropriate for classifying clones about whose origin we have not the slightest idea. Other criteria must therefore be sought.

Classification of organisms consists of two things: one, the analysis of individuals into basic units and, two, the synthesis of these units into like groups. The analysis, I think, presents no great problems with plant viruses, but the synthesis does. Whether or not our units should be called species, is something I shall not pursue to-day. I shall simply follow Mayr and call the unit a 'collective species', by which I shall mean a collection of clones that have many features in common, but differ in some, the most frequent difference so far detected (probably only because it is the easiest to detect) being in pathogenicity. Later work may show that they are true species, in the sense now customarily used for organisms, meaning that the clones of one 'collective species' will inter-breed, but will not cross with clones of another 'collective species'. Recent work with bacteriophages suggests that, when related clones multiply in one cell, they can exchange genetic characters, and techniques may be developed in the future to test whether or not clones of plant viruses can also do so. If they can, we shall have an objective and generally acceptable test for deciding inclusion in a species, but that time still seems far off. Until then we must use some other criterion, preferably one that is also objective, even though we are doubtful of the relationship it discloses. The criterion that seems to me by far the most valuable is the sharing of common antigens. All the viruses so far studied that have been found to be related serologically have also been found to resemble one another in many other intrinsic properties. They have similar sizes and shapes, similar stabilities *in vitro* and similar gross chemical constitutions.

Our first step in classification should be to select specific clones as types of named 'collective species', such as tobacco mosaic, potato X, potato Y, and so on, and antisera should be prepared against each type. Then, by testing individual virus clones against the type antisera, the clones can be allotted to 'collective species' according to the antiserum with which they react. This would be an immense step forward; it would get rid of many synonyms that now plague the literature, and it would show which of the 'new' viruses that are continually being recorded are really new and which are related to already named types. Indeed, it might so decrease the numbers of 'recognized' viruses, that the need for further classification might seem less urgent than it now does.

Unfortunately, serological techniques, although readily applicable to many plant viruses, are not applicable to all. This does not necessarily mean that some plant viruses are not antigenic; more likely it means only that they are too readily inactivated, or occur in too small amounts, to be studied by current serological techniques. No doubt refinements of these techniques will ultimately allow many viruses that are now not amenable to serological study to be handled successfully. There is, though, no need to wait for these refinements before attempting to allot many of these viruses to provisional 'collective species'. Another criterion can be applied; it is to test whether clones can interfere with each other's multiplication in susceptible plants, a feature that seems to be closely tied to serological relationship. Viruses that are serologically unrelated to one another usually fail to depress the multiplication of

each other, and plants infected simultaneously with two such viruses usually show more severe diseases, and often lesions of quite a different type, from those produced by either virus alone. By contrast, if two serologically related viruses are inoculated simultaneously to a plant, they depress each other's multiplication, and the resulting symptoms are usually intermediate between those caused by either alone. Also, a plant already fully invaded by one virus, resists invasion by a second that is serologically related, but not by one that is serologically unrelated. The mechanism underlying the resistance is unknown, but this need not concern us to-day, when the only point of importance is that it is in some way correlated with the antigenic structure of the virus particles and so can be used as a supplementary test for allocating virus isolates to 'collective species'.

It is too much to expect that the plant protection test will always give such unequivocal results as do serological tests, but that it will sometimes fail is no reason for not using it when it succeeds. It will probably give equivocal or false results when the two viruses being tested are only remotely related serologically, for, with only one exception as yet, viruses that share many antigens have protected plants against one another unequivocally, whereas the protection has become increasingly feeble as the number of common antigens decreases. This need not be regarded simply as a deficiency of the test; if carefully used it may be an added value, for it may indicate degrees of relationship between clones. Certainly serological tests seem able to do this. Plant viruses are multiple antigens, and individual clones of a 'collective species' often contain different numbers of common antigens. If it is reasonable to assume that all serologically related clones derive from one original source, those that share many antigens, or that completely protect plants against one another, are also reasonably regarded as more recently derived from a common stock, than those that share only a few antigens or only slightly interfere with each other's ability to multiply in infected plants. In this connexion it is perhaps worth comment that clones of tobacco mosaic virus that are closely related serologically seem also to have similar amino acid constitutions, isoelectric points, electrophoretic mobilities and resistance to inactivation by ultraviolet radiation, whereas those that contain only a few common antigens often differ considerably in such properties. As those with few common antigens also often have widely differing host ranges, the inference is that they have been evolving in isolation from one another.

There is, then, the possibility that within 'collective species' clones might be arranged in some sequence that reflects phylogeny, but beyond this it seems impossible to try to use evolution as a basis for grouping. All the plant viruses that have so far been purified have been found to be nucleoproteins, and their origins are wholly obscure. Each 'collective species' seems to have an equal chance of deriving from a higher plant, a micro-organism, an insect or any other kind of organism, for nucleoproteins are components of all living cells. The fact that we cannot group our 'collective species' by inferred phylogeny is one of the reasons that makes me strongly oppose the use of Linnaean binomial names for plant viruses. These names not only demand

identification at the species level, which I hope I have shown can be done, but the arrangement of species into genera, and the word genus to a modern taxonomist suggests a group of phylogenetically related species that is clearly separated from other genera.

The 'collective species' can, of course, be grouped on criteria other than phylogeny, but then there must be no pretence that the categories do what genera and families do for organisms. The obvious first choice as a criterion is morphology, and present evidence from electron microscopy suggests that three categories, corresponding roughly to bacilli, spirochaetes and cocci, could be made depending on whether the particles are seemingly rigid rods, flexible filaments or spheres. Whether any significance should be given to size, is anybody's guess, for the chemical constitution of no large virus is yet known. If large size should be found to reflect greater chemical complexity, then we shall have a further criterion for grouping according to constitution. At present, however, all plant viruses whose constitution has been determined contain only ribose nucleic acid and protein, and the main difference between 'collective species' is the relative proportions of the two components. It may be taxonomically significant, and a prop to using shape as a criterion for grouping, that, of the viruses so far analysed, those with elongated particles all have the same ratio of nucleic acid to protein, whereas those with spherical particles all have three or more times as much nucleic acid. Although chemistry is not now particularly helpful taxonomically, it can be expected to become so in the future, for if, as is likely, the grouping of viruses is to be achieved on details of morphology, these details are most likely to be shown by the techniques of protein chemistry.

Many plant viruses are not now amenable to study by the electron microscope and so they cannot be grouped according to their gross shapes. Some of these cause similar diseases (the 'yellows' type) in many different hosts, and they are transmitted similarly by leaf-hoppers. *Faute de mieux* these might temporarily be grouped because of such behaviour, but the groupings should be abandoned as soon as such extrinsic characters can be replaced by intrinsic ones. There are too many examples already known of individual clones of one 'collective species' differing widely in pathogenicity and in ability to be transmitted by a given insect, to place any taxonomic reliance on such characters. The main value of pathogenicity and transmission by a vector is not for indicating relationships, but for showing differences between clones of one 'collective species' that otherwise would seem identical.

DISCUSSION

By A. FELIX, *Lister Institute, London*

My remarks are provoked by Dr Andrewes's and Dr Bawden's relapses into the bad habit of using observations on bacteriophages in order to explain some of the difficulties with animal and plant viruses. In support of my own views

I quote from Burnet's recent article in the *Med. J. Aust.* (28 November 1953, p. 809): 'However, I am one of those who in the last year or two have come to believe that the viruses responsible for human and animal disease are not so close to the bacterial viruses as we once thought.' Dr Bawden has been too optimistic about serology and too pessimistic about chemistry in their respective roles as tools in virology. So far as bacteriophages are concerned 'protection tests' or 'interference tests' certainly are no proof of identity of, or even similarity in, antigenic composition.

The Use of Serology in the Classification of Micro-organisms

By P. M. FRANCES SHATTOCK

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In considering the classification of micro-organisms, and of bacteria in particular, one is confronted by two opposing points of view; one, that they should be classified according to their natural relationships; the other, that a natural classification is unimportant, and that all that is needed are keys by which micro-organisms can be identified simply and unequivocally. In agreeing that a natural classification is desirable it must be admitted that this cannot be achieved until there is available a great deal more information upon which to base it. I therefore consider that the first aim should be to build up a system of determinative keys which take full account of the broader fundamental relationships and from these a natural classification might in time be evolved. With this approach in mind, I am convinced that serological techniques could, with great benefit, be more generally used in the systematic study of all groups of micro-organisms. The high specificity of serological reactions is determined by the chemical nature of the antigen, and it is possible to detect differences between complex molecules, particularly proteins, which cannot yet be distinguished by chemical analysis. Serology is therefore a delicate tool for comparing and contrasting antigenic components of the microbial cell, providing information of use both in identification and classification.

This is well illustrated by reference to the genus *Streptococcus*. The classification of the members of this genus, or indeed their unequivocal identification had been extremely difficult. Some species had been well characterized by cultural and physiological tests, but it was not until Lancefield (1933) used serological methods on a collection of strains from a wide variety of sources that the beginning of some kind of order was established. In this genus, *Streptococcus*, we have an example of serological studies which not only detect minute differences between strains but give a broader picture which shows the division of the members into well-defined groups on the basis of specific group antigens. Finally, there is revealed a nucleoprotein antigen shared by streptococci, staphylococci and pneumococci, and one must assume that this wider association is no accident and that the common nucleoprotein does in fact represent a close and possibly natural relationship between these Gram-positive cocci.

A further important example of the use of serology in elucidating broad relationships is provided by the large group of bacteria classified (*Bergey's Manual*, 1948) as the family Enterobacteriaceae. The serological reactions of this family, particularly of the intestinal and allied members, have been more widely studied than any other group of comparable size. Not only did the

pioneer work of Weil & Felix (1920) and of Bruce White (1926) on *Salmonella* provide a background for the later very extensive serological investigations in this genus and with other members of the family, but it was in this family that the classical work of Smith & Reagh (1903), Weil & Felix (1917) and Arkwright (1921) demonstrated the possibility of using serological methods to reveal something of the architecture of the bacterial cell. From studies mainly initiated by epidemiological considerations, a picture of the antigenic structure of the Enterobacteriaceae is being built and although not yet complete, a general pattern is becoming evident. The primary division into genera is made on physiological characters (*Bergey's Manual*, 1948). Whilst serological analysis of the more superficial antigens associated with flagella and capsules has detected slight differences between strains, broader groupings within each genus is possible on the basis of somatic antigens. Furthermore, the sharing of some antigens between genera, e.g. *Escherichia* and *Klebsiella* (Kauffmann, 1949), supports the view that not only are the members of this family closely related but they do in fact form a continuous series.

By analogy with serological observations on the organisms of the family Enterobacteriaceae and on streptococci, pneumococci and staphylococci, the wider or group relationships are generally attributed to the less superficial antigenic components of the cell. This appears to be the case with the genus *Clostridium* in which it has been established that motile species possess flagellar (H) antigens and somatic (O) antigens similar to those described for many aerobic bacteria and it is possible that here also wider divisions may be based on somatic antigens, e.g. *C. tetani* can be divided by flagellar antigens into at least ten types all of which possess a common somatic antigen. However, there is as yet no complete serological survey of this genus, and the picture is so complicated that no definite conclusions can be drawn. Many members of the genus *Clostridium* are characterized by the toxins they produce and serological techniques have been widely used in their identification. However, as Prof. Oakley has already pointed out in this discussion, toxin production is not a sufficiently constant character to provide a basis for classification.

Serological methods have been used to solve practical problems in various fields of microbiology, but comprehensive studies upon which classifications could be based, have been made in only a small minority of bacterial groups. In some cases serological studies have failed to provide a basis for classification and there are no doubt many reasons for such failures. In this connexion the choice of serological techniques may be an important factor. The early success of agglutination reactions in effecting divisions within the genus *Salmonella* encouraged their wide application, but the broad divisions obtained with *Salmonella* on the basis of somatic agglutination were not paralleled for example in the genus *Streptococcus*. Here agglutination techniques, whilst providing a means of identifying individual strains and being of particular use in epidemiological studies of collections of organisms from limited sources (e.g. Griffith, 1927, 1934; Stableforth, 1932), failed as a basis for broader studies (Hucker, 1932). Lancefield (1933), however, showed that antigens

upon which broader groupings could be based were indeed present in streptococci. In agglutination reactions these group antigens are masked by surface antigens which possess narrow specificities. Group antigens can readily be extracted from the cell and their group specificity demonstrated by precipitin techniques.

The agglutination methods generally used in the antigenic analysis of *Salmonella* species do not always give satisfactory results even with other Gram-negative motile rods. Difficulties have been encountered for example with *Pseudomonas aeruginosa* in the preparation, by ethanol treatment, of suspensions for somatic (O) agglutinations (Mayr-Harting, 1948; van den Ende, 1952). Van den Ende solved this problem of technique by preparing trichloroacetic acid extracts, and was able by precipitin tests to make a serological grouping of his strains.

The importance of the choice of antigenic material can also be illustrated by reference to the genus *Bacillus*. Whilst there has been no published systematic study of the serology of this genus, it seems that in the vegetative cell neither somatic nor flagellar antigens form a suitable basis for species differentiation. However, there is a strong indication that spore antigens may well form the basis for a division of species in agreement with physiological studies (e.g. Lamana 1940*a, b*; Davies, 1951). Davies found spore antigens of *Bacillus polymyxa* to be species-specific and later unpublished work by Miss S. N. Davies & Mr H. Proom (personal communication) with various *Bacillus* species, clearly defined by other methods, appears to confirm the species-specificity of the spore antigens.

Identification of species within the genus *Lactobacillus* has been another notoriously difficult problem, and until recently serological studies have done little to clarify the picture. The reasons for these failures are those so often found in investigations of this description. Most workers have confined their studies to investigating the more superficial antigens by agglutination techniques, and mainly with strains from a limited number of sources, thus making it impossible to assess the wider significance of their results. Recently Sharpe (1955), who used precipitin techniques and HCl extracts of lactobacilli, has succeeded in grouping a large and comprehensive collection of named strains and fresh isolates. The serological groups thus defined are in general agreement with certain groupings based on physiological characters (Briggs, 1953), thus emphasizing the significance of the serological findings.

In considering the application of serological techniques to problems of bacterial taxonomy one is faced with the question of deciding what weight should be given to antigenic structure as compared with morphology and physiology. In my opinion the answer must await the results of a greater number of comprehensive studies on a wide variety of micro-organisms; it might then be possible to draw general conclusions on the taxonomic significance of antigenic structure. Nevertheless, it would be well to make use of all the information at present available in an attempt to piece together the jig-saw puzzle. The great difficulty in organizing a logical nomenclature is the lack, even now, of an unequivocal definition of species. This has resulted over

the years in an illogical granting of species rank even within the same genus. Most people who have tried to assess the place of serological studies in the study of bacterial taxonomy would agree that the greatest significance should be placed on broad groupings, and that the large numbers of highly specific reactions often elicited by surface antigens in particular, should not alone be a basis for naming species. The consigning of species names to an almost infinite number of serotypes in the genus *Salmonella* is an extreme example of a great lack of discrimination in this respect. The Enterobacteriaceae subcommittee of the Nomenclature Committee of the International Association of Microbiologists seek to retrieve this position by recommending (*Int. Bull. Bact. Nomen. Tax.*, 1954) that all new serological types of *Salmonella* should be described by formula only and not by name.

Whilst deploring the giving of species names solely on the basis of type antigens, a knowledge of the distribution of serotypes within a particular group may well help the taxonomist to retain or reject some species names already established by other criteria. In this connexion serological typing of streptococci of group D provides information by which species names originally given on physiological grounds can be assessed (Sharpe & Shattock, 1952).

With micro-organisms such as viruses, rickettsias and the pleuropneumonia group where morphological or physiological characters cannot readily be used as a basis for classification, serological studies play an even more important role than they do in the classification of those organisms in which both morphology and physiology can be used more easily. Serological reactions of animal viruses have been very extensively studied in relation to diagnosis and immunity and many antigenic relationships have been discovered which are in agreement with other properties. At the 5th International Congress of Microbiology the Virus subcommittee, in agreeing on the criteria upon which the classification of animal viruses should be based, placed immunological properties high on this list (Andrewes, 1952). With plant viruses, also, serological studies have helped in elucidating relationships, and Bawden (1950) thought that serological criteria would form a sound basis for their classification. With bacteriophages there is no doubt that serology is an excellent basis for classification. For example, Burnett (1933) demonstrated the value of serological relationships in classifying the coli-dysentery group of phages and this classification agreed well with other characters, namely size of phage particle and plaque morphology. The T-series of coli-phages has also been clearly divided, the original seven phages of this series falling into four distinct and unrelated serological groups in agreement with morphological differences (Delbrück, 1946). The reliability of serological reactions in the classification of phages is such that Adams (1953), in discussing criteria for a biological classification of bacterial viruses, regarded serological criteria as of first importance.

In this meeting many methods of approach to the problem of microbial classification have been discussed and it will be recognized that it is not easy to assess the relative value of these various methods in the classification of micro-organisms. Indeed it is all too evident that one comprehensive set of

rules to meet all requirements cannot be formulated. I would submit, however, that the use of serological methods, particularly when supplemented by the chemical characterization of antigens, cannot fail to provide invaluable information to help in the gradual building up of a classification based on fundamental relationships.

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DISCUSSION

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My work on the serological classification of the lactobacilli, mentioned by Dr Shattock, was undertaken not only for its scientific interest, but because the identification of these organisms is of great practical importance.

The collection of organisms used in the serological work comprised strains of all available species, and freshly isolated strains from many different sources. Antisera were prepared against a number of strains. Using HCl extracts and precipitin tests it was possible to place 70 % of the 442 strains examined in six groups and one subgroup. Briggs (1953), working independently on the same collection of organisms, classified most of the strains in a number of physiological groups, with which my serological groups agreed. Wheeler (1955*a, b*) has since extended the work on the physiological characteristics of lactobacilli, and her findings also substantiate the serological groups. A serological classification of many strains of lactobacilli correlated with their physiological characteristics has thus been established.

The lactobacilli appear to possess antigens analogous to those of the streptococci, and this serological classification has been based on them (Sharpe, 1955).

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DISCUSSION

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We have heard various bacteriological criteria discussed in relation to their value in classification. It is obvious that these criteria, which we will refer to as disciplines, have been used for all families of bacteria, but that the importance of any one discipline varies with the family under investigation,

and that all disciplines fail to give a clear-cut answer at one time or another. The next point is that the disciplines are used in a recognized order, an order which for all practical purposes repeats the history of bacteriological research, a sort of phylogeny of the science of bacteriology. This orderly approach is exemplified in the family Enterobacteriaceae where the colonial appearance and morphology are noted, followed by the biochemical tests, then the serology giving the antigenic analysis which may be followed by special biochemical tests and by phage typing. The results of the disciplines used in this order are the basis of classification of the Enterobacteriaceae. For various reasons, we have found it necessary to make certain investigations in which we have not followed the usual order of disciplines. We have noted morphology and colonial appearance, jumped to serology, then returned to the biochemical tests. This approach has given some interesting results, of which the following is an example.

In 1949 a strain, E. 1073₍₂₎, was isolated from a case of infantile gastro-enteritis, and used for the preparation of an antiserum. The antiserum was then used in the investigation of faeces and other material, particularly from cases of gastro-enteritis of infants. By this means, five strains of the same serotype were isolated. In 1952 Dr Patricia Carpenter sent three cultures isolated from the faeces of adults with diarrhoea, which were found to have the same surface and somatic antigens as E. 1073₍₂₎. One was used for the preparation of an antiserum. Some time later, Dr H. Seeliger sent four strains, and the homologous antiserum of one, which he had isolated from an outbreak of gastro-enteritis in adults, and which he labelled 'Katwijk' (Seeliger, 1954). Through the kindness of Brigadier J. S. K. Boyd we also received strains isolated by Dr J. H. Bekker in Holland, which had been investigated by Dr W. H. Ewing, of Chamblee, Georgia, U.S.A. Table 1 shows the results of

Table 1. *Agglutinin titres before and after cross-absorption tests*

Serum	Antigens*	
	'K'	'O'
E. 1073 ₍₂₎		
Unabsorbed	1,600	25,000
Absorbed with E. 1073 ₍₂₎ , or Katwijk, or Carpenter	< 50	< 100
Katwijk		
Unabsorbed	400	3,200
Absorbed with E. 1073 ₍₂₎ , or Katwijk, or Carpenter	< 50	< 100
Carpenter		
Unabsorbed	1,600	6,400
Absorbed with E. 1073 ₍₂₎ , or Katwijk, or Carpenter	< 50	< 100

* Identical results were obtained with antigens E. 1073₍₂₎, Katwijk and Carpenter.

direct titration and agglutinin absorption of the three sera by the three strains, which proves that all three have identical somatic and surface antigens.

A total of fifteen strains has been examined, all of which belong to this serotype. The biochemical reactions of these strains, which fall into three

groups, are given in Table 2. All strains, other than E. 1073₍₂₎, and two strains of the same biochemical type, were non-motile. The biochemical type of Carpenter (Table 2) might well be classified as a shigella. In fact, Seeliger points out that a serologically identical strain isolated in Italy was described as such by Cefalù & Gullotti (1953). The latter also demonstrated that their

Table 2. *Biochemical results*

Strain	Lactose	Glucose, maltose, mannitol	Sucrose, salicin, dulcitol	MacConkey broth 44°	Country of origin and no. of strains
E. 1073 ₍₃₎	AG	AG	AG late	AG	Germany 1 Great Britain 5
Katwijk	A	A	—	A	Germany 3 Great Britain 1
Carpenter	— (21 days)	A	—	—	Holland 2 Great Britain 3

All strains were MR +, VP —, indole +, urease —, citrate —, inositol —. A, acid; AG, acid + gas.

organism, Ca/792, was related antigenically to both *Shigella* serotype 425 and *Escherichia coli* 0.28. Seeliger found an additional relationship to *E. coli* 0.42. We were able to confirm the relationships to the *E. coli* 'O' antigens, and also found that two of the motile strains had the flagella antigens H₄ and H₃₂, respectively, of *E. coli*, but were unable to identify the 'H' antigen of the third strain. These results show that these organisms can be classified within the *Escherichia* group (Kauffmann, 1954). This example is cited as a plea for a reasonable approach to classification, and to show that the value attached to a particular discipline may vary in relation to the results from other disciplines.

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Bacteriophage and Bacterial Classification

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Burnet (1933), and more recently Adams (1952), have discussed the classification of certain phages, and have shown that some of their characters are highly correlated with each other, and are therefore criteria of value in the classification of phages; the value of such a classification is not diminished by our ignorance of the evolutionary origins of the phages.

Bacteriophage is of interest in the study of the classification of bacteria. A particular phage will attack and lyse only a characteristic, limited, range of bacterial strains, and such susceptible strains, able to support the multiplication of a particular phage, are always more or less closely related to one another. So that to show that two bacterial strains are both sensitive to a single phage may give information of value as to their taxonomic relationship.

Phages show varying degrees of specificity, ranging from those which seem to attack but a single strain, to others which attack even strains of different genera. Phages which attack some, but not all, strains of a species have been extensively used for phage-typing; I shall not discuss these phages, but shall consider phages that are of value in defining a bacterial species, genus or larger group.

In the case of a species which is already fairly well defined by several correlated characters, the testing of a number of phages on a group of strains of that species and of related species often shows the existence of 'species-specific' phages, that is phages which attack all (or nearly all) strains of one species, and no strains of any other species. The existence of such phages is evidence of the value of the criteria used to define the species. Such species-specific phages have been used for the routine identification of strains from clinical material, and Katznelson & Sutton (1951) have shown that by their use it is even possible to test for the presence of a particular bacterium in a sample without prior isolation of strains. They were concerned with testing seeds for the presence of the plant pathogen *Pseudomonas phaseolicola*, a bacterium for which they had isolated a species-specific phage; a small amount of this phage was added to a mash of the seeds to be examined; an increase in the titre of the phage on incubation showed that the host bacterium was present. Species-specific phages would probably be more widely used in routine bacteriology if there were some central collection from which they could easily be obtained.

In the more fundamental problem of defining a bacterial species, phages may be of great value. For instance, Smith, Gordon & Clark (1952), in their work on the aerobic mesophilic spore-bearers, examined a large number of spore-bearers, using many different biochemical and other tests, and also

determined the sensitivity of their strains to various phages isolated from soil. They were able to redefine certain species in terms of bacterial characters that were highly correlated with each other, and they found that some of their phages were specific for the species thus delimited. For instance, one phage attacked all of 145 strains of *Bacillus cereus*, another attacked all of 70 strains of *B. pumilus* and a third attacked 83 % of 90 strains of *B. megaterium*; so far as they were tested none of these phages attacked any strains belonging to related species.

A bacterial character is of value as a classificatory criterion to the extent that it correlates with other characters; and in the work of Smith *et al.* the high correlation of phage sensitivity with other characters (e.g. biochemical and morphological) showed the value of both phage reaction and these other characters in the classification of the genus *Bacillus*.

The bacterial character likely to be of use to the taxonomist is the susceptibility of the bacterium to lysis by the phage (or the ability of the bacterium to form a lysogenic system with the phage). The mere absorption of a phage is not likely to be a good criterion, for it is known that some phages may be non-specifically absorbed, e.g. by glass under certain conditions; also, particular antigens, which probably form the point of primary absorption, are known to be widely distributed, so that a certain antigen may recur in two bacterial species which are by no means closely related. Rakietsen & Rakietsen (1937) found that a staphylococcus phage was absorbed by a strain of *Bacillus subtilis*, though the latter was not lysed by the phage; they found some evidence for the existence of a common antigen in the two strains.

A phage-sensitive strain can commonly become resistant to a phage by a single mutational step; or it may in some cases acquire resistance by becoming lysogenic. It is, therefore, not surprising to find that phages which attack only strains of a single species often do not attack every strain of that species. Using such a phage, sensitivity identifies an unknown strain as belonging to the species; but resistance does not exclude the identification. That is, only a positive result is of value, as is the case with many other tests, such as the ability of various species to produce characteristic toxins or antigens.

In addition to species-specific phages there have been reported phages which are specific for groups of higher order, such as those we arbitrarily designate as genera. Of greater interest, however, are the phages with an even wider range, since they give indications of the relationships of different genera, a matter which is generally more in dispute than those of species. In the early phage literature there are a number of reports of the 'adaptation' of, for instance, corynebacterium and staphylococcus phages to attack organisms of the *Shigella* group, and so on. However, this early work now seems unconvincing since the experiments did not prove that the 'adapted' phage was in fact derived from the original phage; in the absence of appropriate controls it now seems impossible to be sure that the 'adapted' phage was not a mutant of a phage carried by the new host, or even a laboratory contaminant. I have not been able to find in the literature any indubitable instances of phages

which attack strains not belonging to what I should consider the same family.

The grouping together of the genera *Escherichia*, *Salmonella* and *Shigella* in the family Enterobacteriaceae is confirmed by the reports of numerous phages which attack strains from two or all three of these genera. It is well established that some phages which attack both *Pasteurella pestis* and *P. pseudotuberculosis* also attack some *Shigella* and *Escherichia* strains; and some phages originally described as *Shigella* phages, such as Burnet's phage C16, also attack *P. pestis* (Flu, 1927; Girard, 1943; Flu & Flu, 1946; Lazarus & Gunnison, 1947). To me this indicates fairly close relationship, and suggests that *P. pestis* and *P. pseudotuberculosis* should be included in the family Enterobacteriaceae, and not relegated to a heterogeneous family such as the Parvobacteriaceae of the 6th edition of *Bergey's Manual*; indeed if one compares the morphology, biochemical activities and nutritional requirements of these two species with those of the *Shigella* group it is hard to find any good differentiating criteria. On the other hand, there do not seem to be any phages reported which attack both *P. septica* and also *P. pestis* or *P. pseudotuberculosis*; this casts some doubt on the propriety of placing *P. septica* in the same genus as the other two species, a grouping which has been criticized on other grounds (Girard, 1942).

Though phages whose range encompasses several species of Enterobacteriaceae are common, none of them seems to be known to attack *Proteus* strains; little has been reported about proteus phages, but there is no good evidence that they attack anything but *Proteus* spp. This suggests that *Proteus* is less closely related to *Escherichia*, *Shigella* and *Salmonella* than these latter are related to each other. Here, too, it seems to me that the hint given by the phages is confirmed by other considerations; for *Proteus*, or at any rate *P. mirabilis* and *P. vulgaris*, differ from the typical enterobacters in quite a number of characters, such as their ability to swarm, their very poor growth anaerobically, their high urease activity, their production of phenylpyruvic acid from phenylalanine, their high resistance to many antibiotics and other antibacterial agents and their production of certain amines.

Some effects of phage other than lysis are also of interest to the taxonomist. In the transduction of characters from one strain of *Salmonella* to another it now seems clear that the role of phage is merely that of a vector of host genetic material from one cell to another. The process is essentially equivalent to the transformations of pneumococci, meningococci and *Haemophilus influenzae*, where deoxyribonucleic acid in the free form is effective, no vector being needed. The transformed bacterium, for instance a strain of *Salmonella typhi* which has been given the flagellar antigen *i* of *S. typhimurium*, is in effect a hybrid of two strains, but a hybrid which has derived all but one of its characters from one of the two parents. Our ability to hybridize two bacterial strains in the laboratory has no more (and no fewer) implications for taxonomy than has the artificial hybridization of two higher plants or animals. It does not invalidate a classification which assigns the two parents to separate groups, e.g. species, but it does suggest that the parental strains are fairly

closely related. There is, as yet, no evidence that these or other phenomena of bacterial hybridization occur under natural conditions. It is, therefore, still possible to treat bacteria as organisms which in nature multiply only by fission, without a sexual process or other mechanism of gene interchange; we may then consider all strains of a valid species, genus or larger taxonomic group as members of a clone, all derived from a single common ancestor. If we assume this we may regard any good practical classification (that is one based on multiple correlated characters) as being of necessity an approximation to a phylogenetic classification.

Many of the bacterial strains which we have to classify are lysogenic; indeed in some species it seems probable that all strains are lysogenic. In such species the characters which we record are not those of the bacterium alone, but those of a stable complex of bacterium and phage (in the latent or prophage form). There is some resemblance to the case of the lichens, which were classified before it was known that each was a symbiotic system comprising an alga and a fungus. The presence of latent phage or prophage in a bacterium does not in general seem to produce very striking effects on bacterial characters such as biochemical activities or antigenic constitution. But the recent work on phage-induced toxigenicity in *Corynebacterium diphtheriae* seems to indicate that here the presence of a particular phage in a bacterium causes it to produce toxin, a character of great interest to the taxonomist. Ideally it would be desirable to study the characters of both the bacterium in its original lysogenic state, and after it had ceased to be lysogenic; but usually we have no means of freeing a lysogenic strain of its phage. However, as the lysogenic complex is in many species the modal form, and that which has been moulded by evolutionary forces, our inability to study the non-lysogenic form does not much reduce the value of our present system of classification, nor invalidate it as a representation of natural relationships.

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DISCUSSION

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Identification and grouping by phage is an additional tool to be used in classification in association with the other information we can obtain about an organism.

There are clear-cut patterns exhibited by *Salmonella paratyphi-B* and *S. typhimurium* with the typing phages of Felix & Callow (1943, 1951) that appear to be specific for each serotype and are not shown by any other salmonellas with these sets of phages. The use of patterns is essential because the phages employed are not completely specific for either *S. paratyphi-B* or *S. typhimurium* but will attack other members of the B group or even members of other somatic groups. These patterns, which are an expression of the resistance or sensitivity of the organisms to groups of phages, frequently owe their form to lysogenicity of the strains being examined.

If an organism shows the lytic pattern of a recognized phage type of *Salmonella paratyphi-B* it can be safely accepted as being *S. paratyphi-B* whether it is monophasic or even non-motile. We have encountered a strain with the antigenic formula 4, 5, 12:b-, and the biochemical characteristics of *S. paratyphi-B*, from clinical cases of enteric fever. This strain is obstinately monophasic and, lacking a second flagellar phase, cannot be designated as *S. paratyphi-B* by the serodiagnostic scheme. Bacteriophage typing identifies it as phage type Jersey of the paratyphoid-B bacillus, a monophasic strain of that serotype.

In the same way, monophasic and non-motile strains having the somatic formula 4, 5, 12:-1, 2, 3 and 4, 5, 12:- have been identified as *Salmonella typhimurium*. In one instance, a non-motile variant identified as *S. typhimurium* phage type 4 was later found to be connected with an outbreak caused by a motile strain of the same phage type.

Another example of the use of phage in classification is found in the Vi-phages of the typhoid bacillus (Craigie & Yen, 1938). Vi-phage II, the typing phage, is capable of modification to a minimum of thirty-three different specificities; these constitute the typing phages. According to the lytic pattern they produce on a strain of *Salmonella typhi*, the Vi-typing phages tell us a great deal about the organisms; we can say whether the strain is lysogenic and it is even possible to describe the characteristics of the phage it carries (Anderson & Felix, 1953; Anderson, to be published).

Strains of *Salmonella paratyphi-C*, paracolonic bacilli and *Escherichia coli* possessing the Vi antigen are sensitive to Craigie & Yen's Vi-phages (Nicolle, Rita & Huet, 1951) but require higher concentrations to produce confluent lysis than does *S. typhi*.

The principle of identifying regular lytic patterns—the so-called 'phage types'—in the paratyphoid-B bacillus and *Salmonella typhimurium* can be extended to other salmonellas, as has been shown by Williams Smith (1951*a, b*) with *S. dublin* and *S. thompson*. Once these patterns are established with certainty, the method can be inverted to identify the serotype by the pattern of phage lysis.

Certain characters of some salmonella serotypes are regularly associated with definite phage types. There is, for example, a phage type of *Salmonella typhimurium* that is always anaerogenic. This type was first encountered in 1943 and is still causing outbreaks in different parts of the country.

The only way in which *Salmonella paratyphi-B* can be distinguished from *S. typhimurium* antigenically is by flagellar serology. The phages that effect this distinction, however, do not attack the flagella; their substrate is entirely the soma of the organism. The reasons for the difference in host specificity of these closely related pathogens—man with paratyphoid-B, and rodents with typhimurium—are unknown, but they are certainly a function of the somatic make-up of the organisms, and it is evident that the phages are capable of recognizing somatic differences that are inapparent to the reticulo-endothelial system of the rabbit.

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DISCUSSION

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I congratulate Dr Stocker on his refreshing and original presentation of the subject. I should also like to congratulate the organizers of the symposium on their bold decision to place bacteriophage on the agenda separately from the item 'The classification of viruses'. Dr Stocker did not speak about classifica-

tion of phages but showed that phages can be employed as a good taxonomic agent for the classification of bacteria, leading to a natural phylogenetic system of bacteria. In my view this meant treating phage as an integral part of the genetic make-up of the bacterium, not as a parasite of extrinsic origin as postulated for animal viruses. Dr Stocker very rightly refrained from discussing 'phage-typing'. This is a technique of bacterial identification for a specific purpose, namely epidemiology, and has little to do with bacterial classification. A point Dr Stocker might perhaps have mentioned is the relationship between colicines and phages.

Summing-up

BY N. W. PIRIE

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Two dangers beset discussions on the principles of classification: they may degenerate and deal with the simpler issue of unequivocal labelling or they may become too impractical and get bogged in the philosophy or even metaphysics of classification. Unequivocal labelling is of the greatest importance but, however perfect the system becomes, it does not lead to an intellectual comprehension of the field covered. After a time the growth of knowledge and the accumulation of specimens or phenomena forces people to try to classify. Classification may be arbitrary, as when the names are arranged alphabetically, or it may be quasi-logical; but in the end a system with some sort of logical background is likely to prevail if only because it is easier to remember. Logic involves some arrangement in which things or concepts are associated in the system to an extent that parallels their resemblance to each other. Some would say their natural resemblance, but natural in this context seems pleonastic. Resemblance can, however, depend on many different factors and the judicious selection of relevant resemblances is the essence of any system of classification.

To this extent classification is arbitrary, we classify from a point of view and with an end in view; no system is ever unique, though the practical convenience of some may give them the appearance of uniqueness. In choosing the features on which to base a classification, preference is likely to be given to properties that distinguish the domain that is being classified from all others, or at least to properties with especial prominence in that domain. The most complete system of classification that we know is that of the metabionta and most biologists nowadays accept evolution as the peculiar feature of this domain. There is no reason to think that all organisms evolved from one ancestor, but each phylum presumably did and common ancestry probably goes back much further than that. A good classification of the metabionta should, therefore, make evolutionary sense and many speakers at this symposium have assumed explicitly or tacitly that classifying was the same thing as arranging species in a phylogenetic order. Very few people now dispute this, and these few seem to most of us to be perverse. But at one time their's was the accepted view. The early classifiers, Ray, Tournefort, Linnaeus, and de Candolle, believed in the fixity of species although various evolutionary ideas were already in the air. It is important to remember that Charles Darwin did not originate the idea of evolution; he suggested an important mechanism for what many scientists and even more educated non-scientists thought had happened. But the taxonomists before Darwin would have none of it. Linnaeus believed that the number of species was fixed at the

Creation and that this belief was an essential part of a sensible classificatory system. His system was a *tour de force* of observation and co-ordination with no logical foundation at all unless we choose to look on it as a logical attempt at deductive theopsychology. It is clear, therefore, that there is nothing impossible about classifying a domain before a body of knowledge has been accumulated about it which, in retrospect, seems necessary for a comprehension of the subject. The attempted classification is indeed often the stimulus for the acquisition of this knowledge.

Attempts to classify the metabionta ran long ago into the difficulty that it is not easy to decide what a species is. This difficulty obtruded itself acutely in our discussion of microbial classification and all shades of opinion were expressed between a disbelief in the usefulness of the species concept as total as that of Buffon, and an acceptance as naïve as that of an elementary textbook. The problem of species is primarily one of labelling rather than of classification; if there were no species there would be nothing to classify. So long as labels can be agreed it is not fundamental to the process of classification whether they represent distinct and unconnected entities, or nodes about which individuals group even although there is continuity between the nodes, or arbitrarily chosen type individuals in a continuous distribution without significant crowding at certain points.

The reality of the categories needs a little more attention than it often gets. Only individuals exist and with varying degrees of certainty these are grouped into species according to rules about interbreeding and so on. At this level a type specimen can be kept, but this is no longer possible at the higher stages of a classification. All the members of a genus or order may share an attribute and this may be absent from the members of another order but no type specimen of even a genus any longer exists. According to the phylogenetic view, and this seems the only logical one, all the individuals in a genus share an ancestor which they do not share with individuals in another genus. The same applies, but more distantly, to orders, classes and so on. We must assume that there was once an object (or pair of them) which was this common ancestor. In principle, except when multiplication is by binary fission, it could have been put in a bottle, after reproduction, and kept as a type specimen. But unless that had been done there is no object corresponding to a genus or higher step in classification. The position is nearly as simple even when there is binary fission, because samples of the original culture could, again in principle, be kept frozen for comparison later with the products of evolution. From a phylogenetic standpoint microbial classification will have a completely different certainty if it should prove possible to reconstruct experimentally the presumed course of evolution. The absence of fossils and embryology does not exclude the possibility of establishing a microbial phylogeny and of testing such schemes as that proposed by Bisset, because these organisms multiply rapidly enough to make the experimental approach possible.

As soon as we have decided how many species we are concerned with it is easy to say how many qualities have to be observed for unequivocal labelling; n qualities would ideally permit differentiation between 2^n species. Thus

ten qualities might suffice for 1024 species and we would almost certainly be safe with fourteen or fifteen qualities. No similar *a priori* approach is possible for classification rather than labelling. The number of qualities that have to be observed is certainly larger but it need not be very much larger and with the metabionta the number to choose from is vast. Thus the mammals can be labelled without looking inside, though dissection is necessary for classification. Things are not so easy with bacteria and viruses; a year's work may be needed to get as many pieces of information about a culture as can be got by a glance at a flower. This is the origin of part of our difficulty.

Many different types of information can be, and should be, woven into the classificatory scheme, and there is no obvious advantage in attaching overriding importance to any one type. We may consider host range and effects on the host, analytical composition and enzymic make up, or morphology, or the production of antigens and toxins. The effects on the host are obviously important because these effects are the reason for most of the financial support for microbiology. If they are made paramount, those saprophytes that seldom or never attack a host would not be included and the position of non-virulent strains becomes equivocal. Furthermore, host range produces many surprises. Thus Elrod & Braun (1941) found that the same bacterium was a cause of disease in tobacco plants and several mammals and it is now generally accepted that some plant viruses multiply in their insect vectors. Finally, if we tied microbial classification to the nature of the disease caused, we would stop arguing about microbial classification until we had settled the principles of disease classification. One of the conclusions of that discussion would probably be that diseases could only be satisfactorily classified in terms of the causative organisms. New possibilities arise when the two wings of our subject attack one another. The bacterial viruses could be classified in terms of their hosts or the hosts in terms of their viruses. Stocker's paper shows that the latter is the more productive direction for the argument.

Biochemical resemblances cannot be taken as overriding or we would put tunicates among the plants because of the importance in them of cellulose, and pigeons among the mammals because they secrete milk and the secretion is controlled by a similar hormonal mechanism. The more detail there is in biochemical information the more useful it becomes; Elsdon asked for information about the mechanism of actions and Clarke asked that the statement that acid and gas are produced should be supplemented by information about which acid and which gas. It would be even better if we got rates of production as well, even if it were only an indication of the order, e.g. 1, 100, 10,000 or 1,000,000 molecules per cell per second. This would prevent similar weight being given to the presence of widely different amounts of enzyme.

Morphology and the production of macromolecules with recognizable distinctive properties are less likely to lead classification astray, because, being essentially imprecise qualities, their users are more aware of the existence of gradations and of the way in which a structure or specificity shades into adjacent structures and specificities. Antigenic specificity is particularly interesting; it is a trustworthy guide in one direction. If an organism does

not share an antigen with others it can hardly be classified with them, but the existence of Forssman antigens and similar phenomena abundantly demonstrates that the possession of a common antigen is no evidence for relationship.

Classification presents a slightly different aspect to those with a chemical rather than a purely biological training, partly because of intrinsic differences and partly because chemistry has explored more of the classificatory possibilities. In the same way that evolution is the peculiar feature of the metazoa, the peculiar feature of the simpler chemical substances is that they contain a precise number of atoms arranged in a determinable way (cf. Pirie, 1952). Before Dalton even the first peculiarity was not recognized and when, in the eighteenth century, chemists wearied of elaborate and ambiguous nomenclature—the vitriols, livers, butters and such gems as *spiritus fumans Libavii*—attempts at rationalization followed several lines. The most interesting was made by Bergman (1784) who, 31 years after Linnaeus and also in Uppsala, tried to classify chemicals on Linnaean lines with genera, classes, and so on. To us, thinking of elements, he seems to perpetrate names as absurd as those he was eliminating and we see that he was trying to classify before he knew enough about the properties of the things classified. This is the odd irony of the situation. The simpler a system is, that is the fewer components it has, the more we have to know about it before sensible classification becomes possible. Hence much of Linnaeus' classification stands now because there are so many attributes of the complex organisms that bad pieces of classification stick out obviously. One can get away with worse fits where, as in chemistry, there are fewer obvious properties to look at.

The attempt is relevant to virus classification because viruses are nearly part of chemistry. People with chemical knowledge, on seeing attempts at Linnaean virus classification, are apt to mutter 'Isn't this where we came in?' And indeed it is. Bergman's grand attempt to force chemistry into a Linnaean strait-waistcoat was bound to fail, not so much because it is not that kind of subject, as because he did not know enough about the intrinsic properties of the things being classified. It is important to remember that, although much of chemical nomenclature is binomial, it is not, in most fields, Linnaean because both words in the name carry equal weight. It is as logical to classify all the chlorides together as it is to classify all the sodium salts, whereas nonsense obviously results if we start grouping together all organisms with *versicolor* as their second name. The argument sometimes advanced that viruses may be classified binomially because so many chemicals already are, is therefore fallacious.

We do not yet know what the peculiarities are that make some substances act as viruses. A fully logical classification is not, therefore, possible. But, just as Linnaeus classified before Darwin and some chemical classification was valid before Dalton, so at least parts of the viruses' domain could probably be classified. If we fail it may be because those who try have not Linnaeus's comprehensive grasp of the subject; alternatively it may be because they are not using enough properties. Even to label the 200 odd plant viruses we would have to use eight qualities and for classification probably a dozen or

more. Virus classification is not, therefore, likely to be put on a satisfactory general basis until many more qualities of each are systematically studied.

A few principles of virus classification seem to be clear. Binomial nomenclature of the Linnaean type, in which the first name unites the members of a genus, can only be used rationally to unite those viruses that are likely to have had a common or similar origin. This may permit a few genera to be set up; Andrewes has made a start and so has Bawden though he prefers to say that he is grouping the varieties of a species. These beginnings will be both scientifically illuminating and practically useful. Attempts to carry co-ordination beyond the genus are not likely to succeed until we know whether viruses originated by variation from one another, by degeneration from bacteria, by the undisciplined behaviour of a normal cell component, or by yet another route. Premature attempts will produce such obvious anomalies that they will keep people from realizing that a few pieces of restricted classification are both useful and valid. From such restricted schemes a comprehensive system will grow gradually. This, besides being of importance in microbiology, will probably be of general use because a classification of the smaller viruses may well serve as a stimulus and model for the classification of other proteins.

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The Nutritional Requirements of *Venturia inaequalis*

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SUMMARY: A study of the nutritional requirements of *Venturia inaequalis* showed that this parasitic fungus grew well in a medium containing: K_2HPO_4 , 0.004 M; $MgSO_4 \cdot 7H_2O$, 0.002 M; NH_4NO_3 , 0.0375 M; glucose 4% (w/v); Zn and Mn 2 p.p.m.; thiamine, pyridoxine, nicotinic acid, folic acid, ascorbic acid. Without thiamine practically no growth of the fungus took place; omission of pyridoxine had nearly as adverse an effect. Raffinose and cellobiose were the best carbon sources; glucose also gave a good yield of mycelium. Statistical analysis of factorial experiments indicated that a correct balance between the mineral constituents of the medium and between the carbon and nitrogen sources is essential in order to obtain a high yield of mycelium.

Apple scab is one of the commonest orchard diseases in temperate regions. The disease attacks all parts of the tree and when infection of the fruit is severe the yield of marketable apples may be decreased by as much as 50%. This disease is caused by the fungus *Venturia inaequalis* (Cooke) Winter, an ascomycete. The conidial phase, which is the more important stage from a pathological standpoint, was at one time called *Fusicladium dendriticum* (Wallr.) Fol.; this stage is found on all living parts of the host. The perithecial stage is less noticeable and occurs only on over-wintered fallen leaves.

Most of the work done on *Venturia inaequalis* in the past has concentrated on the genetics of disease resistance and susceptibility. Johnstone (1931) suggested that resistance to the fungus was not due to any single factor and appeared to depend on the physiological relationship between host and parasite, while the work of Keitt & Langford (1941) indicated that it might be connected with the nutrition of the fungus. The nutritional requirements of *V. inaequalis* have not previously been comprehensively investigated; the present paper describes results of work along these lines.

METHODS

Stock cultures of *Venturia inaequalis* were grown on malt-extract agar slopes containing apple leaf decoction; this MEAL medium was prepared as follows. Air-dried 'Bramley's Seedling' apple leaves (25 g.) were boiled in 500 ml. distilled water for 30 min. Malt extract (5 g.) and agar (25 g.) were dissolved in distilled water and the filtered leaf decoction added to this mixture, which was then made up to a final volume of 1 l. The inoculum for all experiments was prepared by adding 15 ml. sterile distilled water to a $6 \times \frac{3}{4}$ in. tube

containing a well-grown culture on agar (30 days old). The surface of the agar was scratched with a sterile needle, and 1 ml. of the resultant mycelial suspension was then used as inoculum for each experimental flask of liquid medium. It was found by experience that the exact amount of the inoculum did not appreciably affect growth.

In the nutritional experiments 30 ml. volumes of medium were used in 150 ml. Erlenmeyer flasks, with five replicates for each variation of medium. Sterile phosphate solutions were added to the flasks after autoclaving. After incubation the mycelial felts were harvested, dried at 95° and weighed, amounts of growth being expressed as mg. dry wt./flask.

RESULTS

Preliminary experiments on MEAL agar showed that 20° was the optimum temperature for mycelial growth which attained a maximum in 30 days. Various simple defined media containing glucose and mineral salts, such as Czapek-Dox or one devised by Ezekiel, Taubenhaus & Fudge (1934) were tried but growth was extremely poor. The best simple defined medium (SD) found contained: K_2HPO_4 , 0.008 M; $MgSO_4 \cdot 7H_2O$, 0.003 M; KCl, 0.002 M; NH_4NO_3 , 0.0125 M; glucose, 4% (w/v); $MnSO_4 \cdot 4H_2O$ and $ZnSO_4 \cdot 7H_2O$ at 2 p.p.m. [Here and throughout the M concentrations refer to the strengths of solutions calculated from the amounts of salts added; they do not refer to undissociated molecules in solution.] Iron was not added to this medium because it was invariably present in the 'Analar' reagents used. Sterile phosphate solution was added aseptically after the rest of the medium had been autoclaved. In this SD medium the weight of mycelium obtained under the standard conditions of incubation was 8.2 mg./flask.

The effect of various vitamins on growth

To improve the growth on SD medium the effect of adding various vitamin supplements was examined. The following substances were tested in the amounts shown ($\mu g./ml.$ final concentration): biotin (0.4), folic acid (2), thiamine (200), pyridoxine (1200), nicotinic acid (600), riboflavin (200), ascorbic acid (16).

Replicate flasks (5) were prepared which contained SD medium + the seven vitamins, or lacking one vitamin omitted in turn from the medium. The effect of adding asparagine was also tested. The results are shown in Table 1. It is seen that the addition of the 7 vitamins to the best mineral medium (SD) increased amount of growth nearly 18 times. The effect of the different vitamins varied considerably. Omission of biotin or riboflavin had least effect and the omission of pyridoxine, folic acid, nicotinic acid, ascorbic acid or thiamine had increasingly greater effects, in that order, in depressing growth. The effect of ascorbic acid is interesting; when it was omitted from the full SDV medium (=SD medium + 7 vitamins) growth was decreased by 75%. This seems to be an unrecorded effect of ascorbic acid on mycelial fungi. Thiamine, ascorbic

acid, nicotinic acid, folic acid and pyridoxine, which had the most marked effect on growth, were added to all subsequent cultures unless otherwise stated. Ammonium nitrate as nitrogen source gave much better growth than asparagine.

Table 1. *The effects of certain vitamins and other constituents of the medium on the growth of Venturia inaequalis*

Mean dry weight mycelium/flask expressed as percentage of the value for growth on complete SDV medium

Medium	Mean dry-wt. (%)	Medium	Mean dry-wt. (%)
Glucose mineral salts (SD)	6	SDV minus biotin	91
SD medium + 7 vitamins (SDV)	100	SDV minus folic acid	58
SDV but 0.0125 M-asparagine*	9	SDV minus thiamine	16
SD but 0.0125 M-asparagine*	25	SDV minus pyridoxine	63
SDV minus NH_4NO_3	2	SDV minus riboflavin	89
SDV minus K_2HPO_4	3	SDV minus nicotinic acid	42
SDV minus MgSO_4	29	SDV minus ascorbic acid	26
SDV minus KCl	86		
SDV minus Fe	100		
SDV minus Mn	92		
SDV minus Zn	64		

* Asparagine replacing NH_4NO_3 .

The effect of the separate medium constituents and the determination of the optimum pH value for growth

Glucose and various mineral constituents were omitted in turn from the SDV medium in another experiment. Omission of ammonium nitrate and potassium hydrogen phosphate resulted in practically no growth, while omission of other minerals, with the exception of iron, appreciably diminished growth (Table 1).

For the determination of the optimum pH value for the growth of *Venturia inaequalis* the basal medium containing NH_4NO_3 , K_2HPO_4 , MgSO_4 , KCl, Mn, Zn, glucose and the five vitamins thiamine, ascorbic acid, nicotinic acid, folic acid, pyridoxine was used. Cultures were grown in this medium adjusted with HCl or Na_2CO_3 to a range of pH values between 2.5 and 7.0 at 0.3 unit intervals. The phosphate solution was also adjusted and added separately to the medium after sterilization. The optimum pH value was found to be 5.8. There was a rapid decrease of growth as the pH value of the medium decreased to 3.4, at which value there was no growth. There was a much slower decrease in growth as the pH value was increased above 5.8; growth was still reasonably good at pH 7.0. In subsequent experiments all media were adjusted to pH 5.8.

The utilization of carbon compounds

The utilization of different carbon compounds by *Venturia inaequalis* was determined. The basal SD medium + the five vitamins was used but glucose was replaced by other carbon compounds at 4% (w/v).

When acids were added the pH was adjusted to 5.8 with sodium carbonate. The yields of organism (dry weight) expressed as percentage of the yield on glucose were as follows: 130–100 %, cellobiose, raffinose; 100–75 %, dextrin, inulin; 75–50 %, fructose, maltose, rhamnose; 50–25 %, sucrose; 25–10 %, sorbitol, salicin; 10–1 %, galactose, mannitol, dulcitol, malate, succinate, fumarate, glycogen, starch; no growth on inositol, tartrate, oxalate, salicylate, citrate or trehalose.

Thus cellobiose, a glucoside, and raffinose, a trisaccharide, gave better yields than glucose. Dextrin, a glucose polysaccharide, and inulin, a fructose polysaccharide, gave quite good yields. The yield with sucrose was unexpectedly low. The pentoses, some of the hexoses and disaccharides and the organic acids were hardly utilized, if at all. It is evident that certain carbohydrates are readily utilizable by *Venturia inaequalis*. With several lines of this fungus Leben & Keitt (1948) found that cellobiose, dextrin, fructose, glucose, maltose, mannitol, mannose, mellibiose, raffinose or sucrose were good sources for all the strains tested, while organic acids were inhibitory or poorly used. The results given here in the main confirm those of Leben & Keitt, although there are important differences. The present experiments, however, are not strictly comparable with those of Leben & Keitt who used a malt-agar basal medium and recorded their results arbitrarily by visual comparison of growth between the tests and controls. In our experiments glucose was the best commonly available carbon compound.

First factorial experiment; variation of amounts of K_2HPO_4 , $MgSO_4$ and KCl added

The basal physical and chemical requirements of a culture medium for *Venturia inaequalis* having been determined, the effect of the balance of the chemicals used in the medium was investigated. The experiments were designed to show statistically whether or not a balance between the salts is necessary for best growth under given conditions. The factorial design of the experiments also determines the direct effect of the individual salts in the medium and the interaction between them simultaneously.

In this experiment K_2HPO_4 and $MgSO_4$ were studied at three concentrations, while KCl was either present or absent; the experiment was planned to allow an analysis of variance to be drawn up. Eighteen possible solutions were set up each in five replicates, giving a total of 90 cultures in any one experiment. Growth was recorded as dry weight of mycelium. All the mixtures had an initial pH value suitable for good growth of this fungus; the acidity uniformly decreased as growth proceeded. The results are shown in Table 2 which also shows the analysis of variance.

There was a significant difference between the highest and lowest concentrations of K_2HPO_4 . There was no significant difference between the three concentrations of $MgSO_4$ but the highest concentration gave the highest mean weight of mycelium. The presence or absence of KCl made practically no difference to the mycelial weight. This does not mean that chloride is not

essential, small amounts were probably present as impurity, but it does indicate that the organism requires little, if any, chlorine. No addition of chloride was necessary under our conditions. Potassium was present in other components of the medium and its addition as KCl had no appreciable effect on growth.

Table 2. *Dry weight of mycelium of Venturia inaequalis after 30 days growth at 20° on media with different concentrations of certain components*

Concentrations of K_2HPO_4 : P_1 , P_2 , $P_3=0.004M$, $0.008M$, $0.012M$; of $MgSO_4$: Mg_1 , Mg_2 , $Mg_3=0.0015M$, $0.003M$, $0.006M$; of KCl: $0.002M$ or none. Difference required between weights for odds of 99:1 = 6.2 mg. for K_2HPO_4 and $MgSO_4$ and 5.1 mg. for KCl.

No KCl					0.002M-KCl				
	Mg_1	Mg_2	Mg_3	Mean		Mg_1	Mg_2	Mg_3	Mean
P_1	60	43	19	41	P_1	54	39	23	39
P_2	25	35	46	35	P_2	20	41	36	32
P_3	27	36	25	29	P_3	36	26	42	35
Mean	37	38	30		Mean	37	35	34	

General mean = 35 mg. dry wt./flask

General mean = 35 mg. dry wt./flask

Analysis of variance

(Required 'F' and 't' values taken from Snedecor's tables (1934).)

Variance	D.F.	Sum of Squares	Mean Square	F Required odds		
				Found	99:1	19:1
Total	89	16,120	181.13	—	—	—
K_2HPO_4	2	899.99	449.99	5.397	4.88	3.11
$MgSO_4$	2	505.67	252.84	3.033	4.88	3.11
KCl	1	0.0587	0.059	—	—	—
$K_2HPO_4 \times MgSO_4$	4	7,899.78	1,974.94	23.689	3.56	2.49
$K_2HPO_4 \times KCl$	2	297.67	148.84	1.785	4.88	3.11
$MgSO_4 \times KCl$	2	181.40	90.7	1.088	4.88	3.11
2nd order interaction error	76	6,335.85	83.7	—	—	—

The analysis of variance shows the interaction of K_2HPO_4 and $MgSO_4$ to be highly significant. The opposite effect was obtained when the concentration of $MgSO_4$ was increased with the low concentration of K_2HPO_4 to that obtained with $0.008M$ - K_2HPO_4 . Thus the mean weight of mycelium decreased as the concentration of $MgSO_4$ increased with $0.004M$ - K_2HPO_4 . With $0.008M$ - K_2HPO_4 the mean weight increased as the concentration of $MgSO_4$ increased. Thus a proper physiological balance between these two salts is important. There was only a slight difference in the mean weights of mycelium for $0.004M$ - K_2HPO_4 with $0.003M$ - $MgSO_4$, and for $0.008M$ - K_2HPO_4 with $0.006M$ - $MgSO_4$ and the concentrations of these two salts in the latter solution were exactly double those of the former, showing that it is not only the concentration of the salts but the balance between them which is important in the nutrition of this fungus. At the highest concentration of K_2HPO_4 , variation in that of $MgSO_4$ had no significant effect, suggesting that the concentration

of the K_2HPO_4 was too high for good growth. The best growth was obtained with the lowest concentration of both salts.

The other interactions, K_2HPO_4 with $MgSO_4$ and KCl, were not significant and hence the results of the second-order interaction were grouped with the error in the analysis of variance. Best growth was obtained with the lower concentrations of K_2HPO_4 and $MgSO_4$, while the addition or omission of KCl was of no consequence. It is evident that the main effect of the individual salts cannot be extended indefinitely because a correct relationship between the phosphate and the magnesium salt must be maintained.

Second factorial experiment; variation of amounts of K_2HPO_4 , $MgSO_4$ and NH_4NO_3 added

A second factorial experiment was carried out in which NH_4NO_3 , K_2HPO_4 and $MgSO_4$ were varied at three concentrations each, KCl being omitted. A preliminary experiment showed that an increase in the concentration of NH_4NO_3 was beneficial; this salt was used at concentrations of 0.0125M, 0.025M and 0.375M. The preceding experiment indicated that K_2HPO_4 should be present at 0.004M or less; it was tested at 0.001, 0.003 and 0.004M; $MgSO_4$ was used at 0.001, 0.0015 and 0.002M. The method of experimentation was exactly as in the previous factorial experiment. The initial pH values of the solutions were between 6.6 and 7.0; in all cases the acidity increased as growth took place. The results are shown in Table 3 which includes the analysis of variance.

The difference between the three concentrations of NH_4NO_3 was not significant, although the highest concentration gave the greater growth; a relatively high concentration of NH_4NO_3 seems to be necessary for this fungus. This fact can be correlated with the observations of Johnstone (1931) that apple trees deficient in nitrogen are more resistant to scab disease. There was a significant difference for the three concentrations of added K_2HPO_4 and the lowest concentration gave the highest mean weight of mycelium. For $MgSO_4$ there was a difference at the highest concentration tested.

The interaction between NH_4NO_3 and K_2HPO_4 was significant. With the lowest concentrations of NH_4NO_3 the mean weight of mycelium decreased as K_2HPO_4 increased from 0.003 to 0.004M. At 0.025M- NH_4NO_3 the mean weight only fell slightly as the concentration of K_2HPO_4 increased. With the highest concentration of NH_4NO_3 a great decrease in the mean weight was found at the highest K_2HPO_4 concentration. The results suggest that at a low concentration of NH_4NO_3 only a low concentration of K_2HPO_4 could be tolerated. At a higher concentration of NH_4NO_3 the concentration of K_2HPO_4 had little effect possibly because the NH_4NO_3 had not yet reached a critical concentration. With the highest concentration of NH_4NO_3 the lowest concentration of K_2HPO_4 gave the best growth.

The interaction between K_2HPO_4 and $MgSO_4$ was significant. At the lowest concentration of K_2HPO_4 the mean weight of fungus decreased as the concentration of $MgSO_4$ increased, but with the highest concentration of K_2HPO_4

the greatest mean weight was produced. Thus, again, a balance between these salts is important, and when the K_2HPO_4 concentration is increased above a certain level that of the $MgSO_4$ must also be increased. The interaction between NH_4NO_3 and $MgSO_4$ was significant. With the low concentration of NH_4NO_3 low $MgSO_4$ was optimal and with the higher NH_4NO_3 levels high $MgSO_4$ was optimal.

Table 3. *Dry weight of mycelium of Venturia inaequalis after 30 days growth at 20° on media with different concentrations of certain components*

Concentrations of K_2HPO_4 : P_1 , P_2 , $P_3=0.002M$, $0.003M$, $0.004M$; of $MgSO_4$: Mg_1 , Mg_2 , $Mg_3=0.001M$, $0.0015M$, $0.002M$; of NH_4NO_3 (N_1 , N_2 , $N_3=0.0125M$, $0.025M$, $0.0375M$). Difference required between weights for odds of 99:1 = 7.2 mg.)

	N_1			N_2			N_3		
	Mg_1	Mg_2	Mg_3	Mg_1	Mg_2	Mg_3	Mg_1	Mg_2	Mg_3
	mg. dry wt./flask			mg. dry wt./flask			mg. dry wt./flask		
P_1	69	60	40	42	34	49	77	73	46
P_2	65	57	47	44	20	47	30	35	41
P_3	24	15	18	31	24	57	23	15	70

Analysis of variance

(Required ' F ' and ' t ' values taken from Snedecor's tables (1934).)

Variance	D.F.	Sum of squares	Mean square	Required odds		
				Found	99:1	19:1
Total	107	46638.81	431.84	—	—	—
NH_4NO_3	2	999.2	499.6	3.81	4.88	3.11
K_2HPO_4	2	10042.73	5021.36	38.29	4.88	3.11
$MgSO_4$	2	1871.84	935.92	7.13	4.88	3.11
$NH_4NO_3 \times K_2HPO_4$	4	8310.07	2077.51	15.84	3.56	2.49
$NH_4NO_3 \times MgSO_4$	4	4712.59	1178.14	8.98	3.56	2.49
$K_2HPO_4 \times MgSO_4$	4	6511.94	1627.98	12.41	3.56	2.49
$K_2HPO_4 \times MgSO_4 \times NH_4NO_3$	8	3568.92	446.11	3.40	2.74	2.06
Error	81	10621.52	131.12	—	—	—

The analysis of variance also shows that the interaction of all three salts was significant. The higher mean weights were obtained with the highest concentration of NH_4NO_3 . There was little difference between the mean weights for $0.0125M$ - NH_4NO_3 , $0.002M$ - K_2HPO_4 and $0.001M$ - $MgSO_4$, and for $0.0375M$ - NH_4NO_3 , $0.004M$ - K_2HPO_4 and $0.002M$ - $MgSO_4$. This again suggests that a balance between the salts is important.

Third factorial experiment; the importance of a balanced solution

The two previous factorial experiments showed that a definite balance was required between the concentrations of K_2HPO_4 and $MgSO_4$, while the second experiment showed a significant second-order interaction between these two salts and NH_4NO_3 . A third experiment was designed to test the change in the amount of growth which might result when the concentrations of the salts were varied while still maintaining the balance between them. The ratio of

$K_2HPO_4:MgSO_4$ was 0.002M:0.001M. The concentration of these salts was decreased to half in one set of solutions and increased twice and then 4 times in other solutions. Each of these solutions was tested with 0.0125, 0.0375 and 0.0625M- NH_4NO_3 . As in previous experiments the carbon source and vitamins were not varied. Thus twelve different solutions were tested each in five replicates; in each case the pH value was adjusted to 5.8. The results are seen in Table 4. The mean weight of mycelium for the lowest concentration of NH_4NO_3 , i.e. 66 mg., was lower than that obtained with the higher concentrations (144 and 135 mg.), but the latter were not significantly different

Table 4. *Growth responses of Venturia inaequalis to different ratios of K_2HPO_4 and $MgSO_4$ with the same balance and different concentrations of NH_4NO_3*

The twelve different solutions numbered in brackets (1)–(12) are referred to in the text below as treatments.

K_2HPO_4 (M)	$MgSO_4$ (M)	NH_4NO_3 (M)		
		0.0125	0.0375	0.0625
		Mean dry-wt. (mg./flask)		
0.001	0.0005	30 (1)	84 (5)	88 (9)
0.002	0.001	50 (2)	139 (6)	129 (10)
0.004	0.002	84 (3)	177 (7)	154 (11)
0.008	0.004	100 (4)	175 (8)	170 (12)

from one another. The highest weight of mycelium was obtained with 0.0375M- NH_4NO_3 . Within each concentration of NH_4NO_3 the mean weights of the five replicates increased as the concentrations of K_2HPO_4 and $MgSO_4$ were increased, although the balance between the salts remained the same. At the lowest concentration of NH_4NO_3 the first three treatments (nos. 1–3) were different from one another, but treatment no. 4 was not significantly different from treatment no. 3. With 0.0375M- NH_4NO_3 the same effect was produced. The three lower concentrations of K_2HPO_4 and $MgSO_4$ gave mean weights different from one another but treatment no. 7 was not significantly different from treatment no. 8. The four treatments with 0.0625M- NH_4NO_3 all gave mean weights different from one another. These results show that the effects of the balance between K_2HPO_4 and $MgSO_4$ only operated after a certain concentration had been reached and then only in the two lower concentrations of NH_4NO_3 , which suggests that this salt must also be in balance with the other two. The highest mean weight of mycelium was produced by the solution containing 0.004M- K_2HPO_4 , 0.002M- $MgSO_4$ and 0.0375M- NH_4NO_3 .

In another experiment the concentration of glucose was varied (1, 3, 4 or 8%), with NH_4NO_3 at 0.0125 or 0.0375M. Mean dry weights of mycelium were taken after 20, 25 and 30 days of growth. In each case high weights were obtained with 8% glucose and 0.0375M- NH_4NO_3 ; but at this concentration of glucose the growth of the fungus became very mucilaginous, perhaps indicating abnormal growth. Glucose at 4% remains that concentration which gives a normal mycelium with the highest dry weight of fungus.

CONCLUSION

It is concluded from this work that a medium containing 0.004M- K_2HPO_4 , 0.002M- $MgSO_4$, 0.0375M- NH_4NO_3 , 4% (w/v) glucose, Zn and Mn at 2 p.p.m. and sufficient amounts of thiamine, pyridoxine, nicotinic acid, folic acid and ascorbic acid, gives a maximum growth in liquid cultures when grown at pH 5.8 and 20° for 30 days. Although raffinose and cellobiose gave greater yields than glucose, the latter remains the common sugar which gives good growth. The factorial experiments support the conclusion of Talley & Blank (1941), who also worked with a parasitic fungus, that a proper balance between the major mineral constituents of the culture medium is necessary. But they do not confirm the conclusions of Fothergill & Raine (1954), who worked with a saprophytic fungus and found that while a balance of salts was important, some other factor (or factors) was also involved in those processes which lead to increases of dry weight of the mycelium. A balance between the carbon and nitrogen sources is also essential.

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The Mechanism of Action of the Fungicide, 2-Heptadecyl-2-imidazoline

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SUMMARY: The hypothesis that the fungicide 2-heptadecyl-2-imidazoline might owe its effectiveness to interference with the synthesis of histidine or purines was tested in experiments with *Sclerotinia fructicola*. The toxicity of the fungicide was significantly decreased by guanine, xanthine, or xanthosine; a number of other related compounds and histidine had no effect.

Annulment of the action of 2-heptadecyl-2-imidazoline by either xanthine or guanine was competitive. The inhibition index for 2-heptadecyl-2-imidazoline/xanthine was 0.000581, while that for 2-heptadecyl-2-imidazoline/guanine was 0.000832. Attempts to demonstrate the accumulation of the purine precursor 5-amino-4-imidazolecarboxamide in culture filtrates of *Sclerotinia fructicola* grown in the presence of the fungicide were negative.

Despite the wide use of many organic fungicides, little information is available concerning their mechanism of action (Horsfall, 1945). The present report is concerned with 2-heptadecyl-2-imidazoline (2-heptadecyl-2-glyoxalidine), the active ingredient of Crag Fruit Fungicide 341, manufactured by the Carbide and Carbon Chemicals Company, New York. This material is widely used in the control of apple scab, caused by *Venturia inaequalis* (Cke.) Wint., and cherry leaf spot, due to *Coccomyces hiemalis* Higgins (Thurston, Harry, Lewis, Groves & Taylor, 1946; Thurston, 1950). It has previously been shown that homologues of 2-imidazoline with side chains differing in length gave dosage-response curves which were essentially parallel, indicating that the antifungal activity was centred in the 2-imidazoline nucleus (Wellman & McCallan, 1946). Attempts to explain fungitoxicity on the basis of surface activity (Miller, McCallan & Weed, 1953) were unsuccessful.

Examination of the structure of 2-heptadecyl-2-imidazoline indicated similarities to certain naturally occurring compounds. The imidazole ring, present in the fungicide, is also to be found in the amino acid histidine, in allantoin, and in the purines and their derivatives. The hypothesis that the fungicide might owe its effectiveness to acting as an inhibitor of one or more of these essential metabolites was tested in a series of experiments, using *Sclerotinia fructicola* (Wint.) Rehm as the test organism.

METHODS

Stock cultures of an original isolate of *Sclerotinia fructicola* were maintained on slants of potato glucose agar at c. 26°. The medium evolved after preliminary work contained (per litre): glucose, 90 g.; L-glutamic acid, 14 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$,

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0.5 g.; KH_2PO_4 , 1.5 g.; K_2HPO_4 , 0.2 g.; FeCl_3 , 5 mg.; thiamine HCl, 1 mg.; pH adjusted to 3.4.

Crag Fruit Fungicide 341 consists of 34 % (w/v) 2-heptadecyl-2-imidazoline acetate and 66 % (w/v) isopropanol. Dilutions were made with distilled water, and the fungicide was added to the basal medium before autoclaving.

Cultures were grown in 125 ml. Erlenmeyer flasks containing 50 ml. medium. The media were sterilized by autoclaving at 15 lb./sq.in. for 20 min. Inoculum was prepared from cultures grown on slants of potato glucose agar for 7–9 days. Culture flasks were inoculated by means of sterile 1 ml. pipettes with a spore suspension made up in basal medium.

Cultures were incubated at *c.* 26° under stationary conditions for 15 days. The mycelial mats were removed by filtration through filter-papers which had been previously dried to constant weight in a desiccator containing 'Drierite' (anhydrous calcium sulphate, W. A. Hammond Drierite Co., Xenia, Ohio). The filter-papers containing mycelial mats were partially dried in an oven at 60°, and the drying to constant weight was completed in a desiccator containing 'Drierite'.

The concentration of purines, purine ribosides, purine ribotides, and other substances tested for possible effectiveness in annulling the action of the fungicide was routinely 1 mg./ml. These chemicals were obtained from General Biochemicals, Inc., Chagrin Falls, Ohio; Nutritional Biochemicals Corp., Cleveland, Ohio; or Dougherty Chemicals, Richmond Hill, N.Y. In experiments on the nature of the annulment, the concentrations of fungicide and other compounds were varied while keeping the fungicide/other compound ratio constant. The inhibition index (Woolley, 1952) was calculated from the concentrations of fungicide and annulling compound which give 50 % inhibition of growth.

Because certain purines were shown to interfere with the function of 2-heptadecyl-2-imidazoline as a fungicide, and because 5-amino-4-imidazolecarboxamide has been shown to be a purine precursor (Stretten & Fox, 1945; Shive, Ackermann, Gordon, Getzendaner & Eakin, 1947), attempts were made to determine whether this compound accumulated in the filtrates of cultures whose growth was partially inhibited by the fungicide. The method used to estimate the imidazolecarboxamide, which is a general one for non-acetylatable diazotizable amines, was that of Bratton & Marshall (1939), modified by Ravel, Eakin & Shive (1948).

RESULTS

In a preliminary experiment it was found that guanine and xanthine were very effective in annulling the action of 2-heptadecyl-2-imidazoline, while adenine, hypoxanthine, uric acid, allantoin, and L-histidine were without effect. A wider range of purines and purine derivatives, including those previously tested, 2:6-diaminopurine, caffeine, theobromine, theophylline, adenosine, guanosine, inosine, xanthosine, adenylic acid, and guanylic acid, were then tested in an experiment in which a 3.9 μM concentration of 2-heptadecyl-2-imidazoline inhibited the growth of *Sclerotinia fructicola*, *c.* 92 %.

Any degree of inhibition less than this may be considered as indicative of annulment of the action of the fungicide. Using this criterion, a significant degree of annulment was found with guanine, inhibition *c.* 57 %; xanthine, inhibition *c.* 3 %; and xanthosine, inhibition *c.* 68 %; but with none of the other compounds tested. 2, 6-Diaminopurine, which is readily employed as a nitrogen source by certain fungi but is inhibitory to others, was strongly inhibitory to the growth of *S. fructicola*.

Thus, of the compounds tested only guanine, xanthine or xanthosine annulled the fungicidal effect of 2-heptadecyl-2-imidazoline with certainty. An experiment was designed to examine the nature of the annulment, and to determine whether purine synthesis was affected. Purine and fungicide concentrations were varied, while the fungicide/purine ratio was kept constant. The fungicide/purine ratio chosen was equivalent to a 3.9 μM concentration of the fungicide and a 4680 μM concentration of the purine. Controls were provided in which the fungicide but no purine was added to the basal medium in the concentration range studied. The results are summarized in Table 1.

Table 1. *The effect of various concentrations of guanine, xanthine, and 2-heptadecyl-2-imidazoline, alone and in combination, upon the growth of Sclerotinia fructicola*

Addition to basal medium	Amount of purine/50 ml. (mg.)	Fungicide (μM)	Mat weight, purine plus fungicide (mg.)	Mat weight, fungicide (mg.)	Growth, percentage of control
None	0	0	749*	—*	100.0†
Guanine	100	7.811	639	33	5.2
	66.7	5.207	790	35	4.4
	50	3.906	612	30	4.9
	33.4	2.603	654	203	31.0
	25	1.953	1079	754	60.6
	20	1.562	843	738	87.5
Xanthine	100	7.811	839	33	3.9
	66.7	5.207	769	35	4.6
	50	3.906	722	30	4.2
	33.4	2.603	784	203	27.2
	25	1.953	1203	754	62.7
	20	1.562	938	738	78.7

* All figures in these columns represent the average of duplicate determinations.

† Amount of growth in the absence of purine, as percentage of amount of growth in presence of purine.

Certain conclusions are evident from the data included in Table 1. In the guanine experiments, in which both the fungicide and guanine concentrations were varied by a factor of five, while the guanine/fungicide ratio was constant, roughly the same amount of growth was found in all instances. The amount of growth is therefore not proportional to either the fungicide concentration or to the guanine concentration, but is dependent on the fungicide/guanine ratio. Similar findings are evident also with xanthine. It may be concluded that annulment of the action of the fungicide by guanine or xanthine is competitive.

The data concerning guanine in Table 1 are presented graphically in Fig. 1.

The inhibition indices characteristic of the annullment of the action of 2-heptadecyl-2-imidazoline upon the growth of *Sclerotinia fructicola* by guanine or xanthine were calculated as 0.000832 and 0.000581 respectively. Guanine was then considerably more effective than xanthine in annulling the action of 2-heptadecyl-2-imidazoline.

It seemed a reasonable inference from the preceding experiments, in which it was shown that certain purines annulled the action of 2-heptadecyl-2-imidazoline upon *Sclerotinia fructicola*, that the fungicide blocks the synthesis of

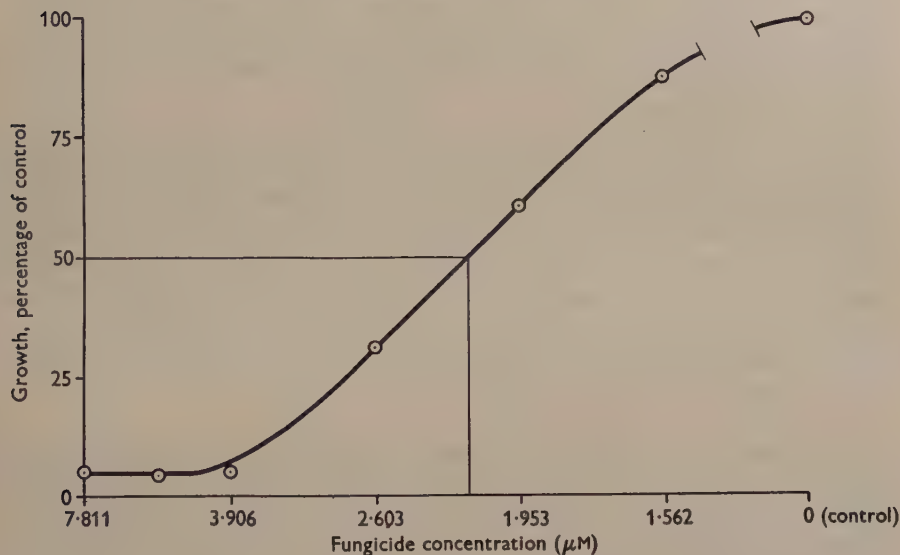


Fig. 1. Growth of *Sclerotinia fructicola* in various concentrations of 2-heptadecyl-2-imidazoline, expressed as a percentage of growth in controls lacking guanine, when compared with growth in a 3.9:4680 fungicide:guanine mixture having the same fungicide concentration as the controls.

these purines, which are essential for growth. If this were so, a purine precursor might accumulate in the filtrates of cultures, the growth of which has been partially inhibited by the fungicide. One such purine precursor already known is 5-amino-4-imidazolecarboxamide (Stetten & Fox, 1945; Shive *et al.* 1947). Experiments were performed in which culture filtrates of *S. fructicola*, partially inhibited by 2-heptadecyl-2-imidazoline, were examined for the presence of such a diazotizable amine by the method of Bratton & Marshall (1939) as modified by Ravel, Eakin & Shive (1948). Culture filtrates of *S. fructicola* grown on the basal medium alone, in the presence of 3.9 μM-2-heptadecyl-2-imidazoline, in the presence of guanine, xanthine, or xanthosine (4680 μM), and in combinations of fungicide and purines, were tested for diazotizable amines with consistently negative results; the method would have indicated *c.* 2.5 μg. 5-amino-4-imidazolecarboxamide/ml.

DISCUSSION

The experiments reported indicate that the fungistatic activity of 2-heptadecyl-2-imidazoline is counteracted by guanine, xanthine, or xanthosine, but not by other purines, purine derivatives, histidine, or allantoin. The compounds annulling the action of the fungicide are closely related structurally, guanine differing from xanthine only in having an amino group in the 2-position. Guanine is easily converted to xanthine in a number of organisms by a simple deamination reaction. The experiments with guanine or xanthine in combination with 2-heptadecyl-2-imidazoline indicate that the latter compound is a competitive inhibitor in the synthesis of these purines. The small inhibition indices indicate the high effectiveness of 2-heptadecyl-2-imidazoline as a purine inhibitor.

The nature of the enzyme or enzymes acted upon by 2-heptadecyl-2-imidazoline was not revealed in these experiments; they might be presumed to function in the biosynthesis of guanine and xanthine. The failure to detect a diazotizable amine does not necessarily indicate that one is not an intermediate in purine synthesis, since it is possible that it is so rapidly converted to other products as to escape detection. It also seems possible, because of the specificity shown in annulling the action of 2-heptadecyl-2-imidazoline, that xanthine and guanine might be formed in *Sclerotinia fructicola* by a route different from that of other purines.

Appreciation is expressed to the Carbide and Carbon Chemicals Co., New York, N.Y., for the supply of Crag Fruit Fungicide 341; to Dr W. Shive, Department of Chemistry, University of Texas, Austin, Texas, for providing 5-amino-4-imidazolecarboxamide; to Dr O. Touster, Department of Biochemistry, Vanderbilt University School of Medicine, for supervising the determinations of diazotizable amines. The material included is from the thesis of the senior author, presented to the Graduate School of Vanderbilt University in partial fulfillment of the requirements for the degree of Master of Arts.

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Lysogenicity in *Xanthomonas pruni*

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SUMMARY: An apparently lysogenic strain of *Xanthomonas pruni* was isolated. A series of experiments was performed to determine whether the isolate was lysogenic or whether it was a case of pseudolysogenesis. The results indicated that it was a true case of lysogenesis. The phage released by the lysogenic organism differed from the one used in its selection. The possible origin of this new phage and its lysogenic host is discussed.

Several types of *Xanthomonas pruni* phage have been characterized by their differences in plaque morphology, host range, serological properties, in burst times, and in burst sizes (Mandell & Eisenstark, 1952, 1953; Kirchner, 1954). In the process of isolating variant hosts which might be resistant to one or more of the phage types, a culture was obtained and designated as H1 5L, which exhibited the possibility of its harbouring of phage. H1 5L was resistant to all of the *X. pruni* phage types. When dilutions of H1 5L were plated out on a susceptible host, H1, plaques appeared, even though no phage had been added. Such action might have resulted from lysogenesis of H1 5L, or as the result of phage mechanically carried by resistant H1 cells (pseudolysogenesis). These situations have been discussed in detail by Lwoff (1953). A series of tests was performed to determine whether the phenomenon was due to actual lysogenesis existing in *X. pruni* or to carried phage.

METHODS

The procedures for plating, titrating phage and preparing media were essentially the same as those described by Adams (1950) for *Escherichia coli* strain B except that plates were incubated at 24° rather than 37°.

The two *Xanthomonas pruni* hosts described in this report are H1 5L, the suspected lysogenic culture, and H1, obtained originally from the American Type Culture Collection, no. 10,016, which is susceptible to all of the phage types that have been isolated. The types of phage used in the *X. pruni* phage work have been designated as Xp1, Xp2, Xp3, Xp4, Xp5 and Xp8. All of these behave as virulent phages. Xp8 is the phage that is released by host H1 5L. The suspected lysogenic culture, H1 5L, was obtained by plating H1 and an excess of Xp4 phage. After incubation, resistant colonies were selected.

The Xp4 stock used in these experiments was obtained originally by selection of a particular plaque type from a mixed population. In order to minimize the presence of mutant forms, Xp4 stocks for each experiment were prepared by selection of isolated typical Xp4 plaques.

The method described by Adams (1950) was followed in the determination of antiserum inactivation constants.

RESULTS

Isolates of suspected lysogenic organisms were restreaked, incubated, harvested and washed in an attempt to rid them of externally carried phage. This procedure was followed through twenty restreakings. Lysis still occurred when re-isolates were plated back on the susceptible host. When susceptible host H1 was exposed to phage Xp1 and similarly treated, it lost phage Xp1 after the second or third restreaking. This is interpreted as evidence that the phage in the suspected lysogenic culture was not carried mechanically; otherwise it would have been lost upon restreaking as was Xp1.

Antiserum will inactivate extracellular phage but not intracellular. Hence, if culture H1 5L is truly lysogenic, it should be possible to recover phage even though the culture has been transferred through an antiserum medium. It was found that after seven transfers through antiserum medium, culture H1 5L continued to release phage.

Cells of H1 5L were plated on plain nutrient agar to get a regular colony count and on nutrient agar seeded with susceptible host to get a plaque count. In the presence of lysogenesis, there would be an approximate 1:1 ratio of colonies to plaques. If the phage were being carried mechanically, the plaque count should exhibit no relationship to the colony count. The data in Table 1 indicate that the plaques probably originated from single lysogenic organisms, rather than from free phage. Although the ratios are not 1:1, there does appear to be a significant relationship between the number of colonies and the number of plaques.

Table 1. *Ratio of colonies to plaques when aliquots of H1 5L were plated out on (1) nutrient agar and (2) nutrient agar seeded with host H1*

Sample no.	Colonies	Plaques	Ratio
1	302	908	1:3.0
2	75	290	1:3.9
3	37	121	1:3.3

An additional step was to make replica plates (Lederberg & Lederberg, 1952) from colonies of H1 5L on to agar plates which had been seeded with susceptible host. A halo developed around each colony that arose on the new plates, indicating lysis. If this were a consequence of carried phage, the results of this replica plating technique would indicate that each bacterial colony had carried phage, an unlikely situation. A more conceivable explanation is that each colony consisted of lysogenic cells.

A comparison of the phage (Xp8) arising from the lysogenic host with the phage (Xp4) which had been added to the host in the original isolation process revealed distinct differences. If Xp8 had been a carried phage, it should have been identical with Xp4, since Xp4 was the only phage known to have been in contact with this host. Xp8 was found to differ from Xp4 in plaque morphology, as may be seen in Pl. 1 A, B. Almost all of the plaques produced by Xp8 were cloudy, small, and irregular, while Xp4 plaques were much larger,

clear, with a distinct halo. In addition to the cloudy plaques, a small number of larger and clear plaques were produced by Xp8. A few may be seen in Pl. 1B. Studies are in progress to determine the origin and significance of this second plaque type.

Xp8 differs from Xp4 in its host range, as may be seen in Table 2. A series of isolates has been collected which are resistant to one or more of the Xp series of phages. These were used to show that the host range of Xp4 and Xp8 differed.

Table 2. *Host range differences*

Host	Xp4 phage	Xp8 phage
H1	Lysed	Lysed
H1 5L	Not lysed	Not lysed
H80	Lysed	Not lysed
H23	Not lysed	Lysed
H8	Not lysed	Lysed
H204	Not lysed	Lysed

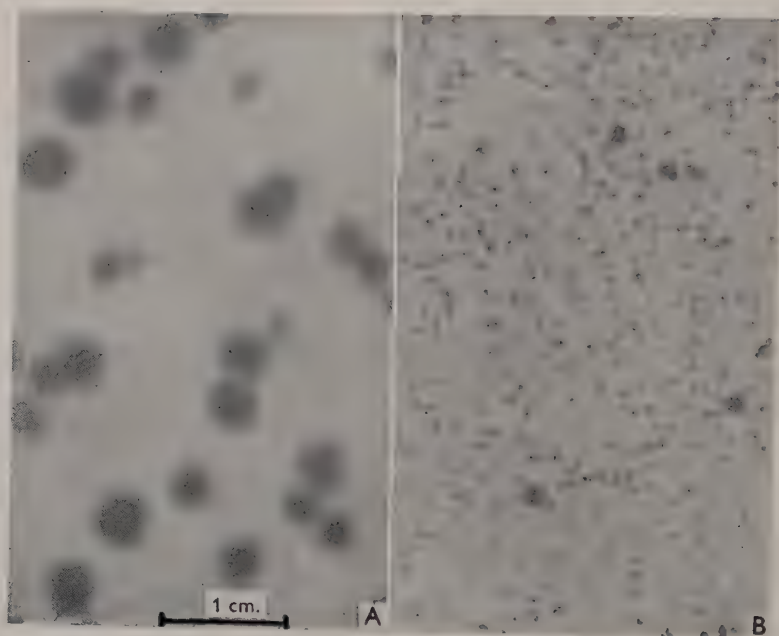
In order to test the possibility that Xp4 stock might contain a small number of contaminant Xp8 phage, Xp4 was plated on host H23, which is resistant to Xp4 but susceptible to Xp8. In the plating of 10^{10} Xp4 phage not a single plaque arose, indicating that if the Xp4 stock was contaminated with Xp8, the contaminant must be present in a frequency less than one out of 10^{10} .

In addition to the above comparisons of Xp4 and Xp8, immunological data were also obtained. Immunologically it was found that Xp4 and Xp8 do not fall into distinct groups, although they are not inactivated at equal rates by Xp4 antiserum. The inactivation constant for the homologous system, Xp4 phage and Xp4 antiserum, was found to be $K=95.6$, whereas the constant for Xp8 phage and Xp4 antiserum was $K=61.5$.

If the phenomenon in question resulted from a carried phage, it should be possible to demonstrate it with any of the Xp phages. H1 5L is resistant to all of the Xp series and if it were merely a selected culture that was carrying phage, it should not make any difference which Xp phage was used in the selection process. However, it was found that Xp4 was the only one of the series that enabled the isolation of H1 5L. In the process of selecting hundreds of resistant organisms, about 25 % of the isolates from Xp4 plates possessed the properties of H1 5L, whereas no isolate from plates seeded with Xp1, Xp2, Xp3 and Xp5 exhibited similar properties.

DISCUSSION

Ordinarily, when dilutions of lysogenic bacteria are plated on a susceptible host, the plaques that arise exhibit a central colony inasmuch as the lysogenic bacterium itself grows into a distinctly visible colony. An occasional organism within a colony bursts and releases phage, hence the plaque around the colony. When H1 5L was plated on H1, central colonies could be seen in only a small percentage of plaques, and then only indistinctly. Possibly the plaque



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XANTHOMONAS PRUNI. PLATE 1

(Facing p. 405)

itself was so turbid and small that the central colony was obscured by this growth throughout the plaque.

The results of this investigation pose several questions relative to the origin of H1 5L (Xp8). Does H1 actually contain a small number of H1 5L (Xp8) cells, and Xp4 merely select these by lysing the remainder of the population? This seems unlikely in the light of the above experiments where Xp1, Xp2, Xp3 and Xp5 all failed to act as selecting agents.

Are some of the Xp4 phage able to behave as temperate phage that invade H1 cells and cause them to be lysogenized? This would seem to be a possible explanation since lysogenization is a common occurrence among other phage-host systems (Lwoff, 1953). However, if this is the case, why is the phage that is released by H1 5L (Xp8) apparently different from Xp4? A possible explanation, that Xp4 is not pure and contains a small proportion of temperate Xp8 phage capable of lysogenizing host cells, seems unlikely since no plaques arose when 10^{10} Xp4 phage were plated out on H23 which is resistant to Xp4 but susceptible to Xp8. It is also possible that Xp8 represents a host-induced modification (Luria, 1953) of Xp4 which actually has changed its characteristics after a multiplication cycle within its host. A third possible explanation is that the *Xanthomonas pruni* cells harbour Xp8 prophage, but that the mature phage are not developed until the bacterial cells are stimulated to do so by Xp4 nucleic acid. These explanations are in need of careful experimental examination.

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EXPLANATION OF PLATE

- A. Plaques produced by Xp4. These are large and clear with a distinct halo.
- B. Plaques produced by Xp8, the bacteriophage released by lysogenic cells of *Xanthomonas pruni*. These are minute and cloudy. There is always a small percentage of larger plaques. A few of these plaques may be seen in the figure.

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The Effect of Enzyme Inhibitors on the Multiplication of T 2 Bacteriophage

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SUMMARY: Screening tests on the inhibition of growth of phage T2r⁺ and of its host, *Escherichia coli* strain B, by a number of enzyme inhibitors, showed that only arsenite, borate and cyanide inhibited the phage at concentrations which were without effect on the phage alone, on its adsorption to bacteria, or on bacterial growth. In single-step experiments, the inhibitory effect of arsenite and cyanide on phage multiplication was maintained for several hours, whereas borate merely lengthened the latent period by a few minutes without decreasing the final titre. Arsenite was still effective as an inhibitor when added half way through the latent period.

Work on the effect of inhibitors on the multiplication of bacterial viruses has been carried out almost since the discovery of bacteriophage; but it is only with the more recent interest in chemotherapy and antibiotics, and with the possibility that results with bacterial viruses might be applicable to other viruses, that it has become extensive. Compounds of many kinds have proved to inhibit virus multiplication but, with few exceptions, their mode of action is obscure. This is perhaps because the compounds were used empirically to prevent virus growth, rather than for studying the inhibitory process. The work of Cohen (Cohen, 1951; Cohen & Roth, 1953; Scott & Cohen, 1953*a, b*) on the metabolic processes of *Escherichia coli* and on the isolation of the enzymes concerned has thrown more light on the mechanisms involved in phage production; but inhibition experiments can play their part in elucidating the process. The results presented in this paper show that substances known to be enzyme inhibitors can inhibit phage production at concentrations which are without effect on bacterial growth, and that the type of inhibition may vary according to the enzymic reaction affected.

METHODS

Media. The liquid medium used (GTP broth) contained 2% (w/v) Difco Bacto Tryptose; 0.2% (w/v) glucose; 0.5% (w/v) NaCl; and 0.01 M-phosphate buffer (pH 7.2).

Both phage and bacteria were diluted in a medium (phosphate saline) containing: 0.08 M-NaCl; 0.01 M-phosphate buffer (pH 7.2); 0.005 M-magnesium chloride; 0.1% (w/v) gelatin.

Bacteriophages. *Escherichia coli* strain B, and the T series of bacteriophages were originally obtained from the late Dr W. J. Elford, F.R.S. The *E. coli* strain B, was subcultured fortnightly on nutrient agar slopes with periodical plating out to test purity of the stock, and isolation of single colonies which

were then tested to ensure that they had the same sensitivity to the T phages as the parent strain.

Concentrated bacteriophage preparations were obtained by the method of Swanstrom & Adams (1951), the final product being dialysed against m/15 phosphate buffer (pH 7.2). Titres were usually in the range $1-5 \times 10^{11}$ particles/ml. In some cases GTP broth lysates of coliphage T2r⁺ were concentrated by a modification of the method of Herriott & Barlow (1952): the lysate was mixed with acid- and alkali-washed Stellafilt (Grade SSS, Paterson Engineering Co. Ltd., 83 Kingsway, London), filtered, cooled to 0° and the pH brought to 3.9-4.0 with N-HCl. The precipitate was then centrifuged down at 0°, suspended in a small volume of saline at 0° and dissolved by addition of the minimum quantity of M-NaHCO₃. After a preliminary titration the product was diluted to a titre of 5×10^{11} with phosphate saline.

In all bacteriophage preparations sterility was maintained with a crystal of thymol.

Bacteriophage counts. These were made in duplicate by the plaque-counting method of Hershey, Kalmanson & Bronfenbrenner (1943), using digest broth solidified with 1.5% (w/v) agar for the base and with 0.75% (w/v) agar for the upper layer.

Bacterial counts. Bacterial growth was followed by viable count or by nephelometer (Evans Electroselenium Ltd.) calibrated against total counts of log phase cultures of *Escherichia coli* strain B. The viable count was always measured when growth was occurring in the presence of an inhibitor.

Inhibitor solutions. Inhibitors were usually dissolved in saline; but 8-hydroxyquinoline was dissolved in ethanol and the solution diluted with saline to below 1% (v/v) ethanol. Sulphanilamide and 2':4-dinitrophenol were dissolved in the minimum of N-NaOH, and arsenite was used either as the salt or by dissolving As₂O₃ in the minimum of N-NaOH. All solutions were brought to pH 7 before use.

SCREENING TESTS

Preliminary experiments were made with a number of substances known to inhibit a range of enzyme reactions, to discover those which would decrease phage yield at levels which were without effect on bacterial growth. An inhibitor which does not completely suppress phage multiplication may (1) increase the length of the latent period; (2) increase the length of the rise period; (3) decrease the final titre; or may produce all three effects in combination. The criterion of inhibition has usually been decrease in final titre, but changes in latent period or increase in titre may be more sensitive indicators, since partial inhibition of an enzymic process essential to phage production might decrease the rate of phage multiplication without decreasing the number of phage particles finally produced. Comparison between the titres of control suspensions of phage and bacteria and those containing inhibitors shortly after the end of the normal latent period will show if any of these types of inhibition is occurring, and also has the advantage that it does not entail the large amount of plating needed to follow growth curves. Experiment showed

that when a log-phase culture of *Escherichia coli* strain B, in GTP broth (5×10^7 organisms/ml.) was infected with an equal number of T2r⁺ particles and titrated at intervals by pipetting samples into phosphate saline at 0° to prevent further multiplication, the phage titre began to rise at 18–19 min. and had increased five- to tenfold by 20–21 min. This system was therefore made the basis of the inhibition test.

A series of tubes containing different concentrations of freshly prepared inhibitor solution (1 ml.) were brought to 37° in a water-bath and to each at 1 min. intervals added 10 ml. of a log-phase culture of *Escherichia coli* strain B, in GTP broth, containing 5×10^7 organisms/ml. as measured by nephelometer. When culture and inhibitor in each tube had been in contact exactly 10 min. 5×10^8 T2r⁺ particles in 0.1 ml. broth were added to it. After 20 min. incubation 0.1 ml. was withdrawn into 20 ml. phosphate saline at 0° to stop the phage multiplication and, after further dilution, assayed for phage. A duplicate set of tubes containing only inhibitor and bacterial culture was set up at the same time, incubated for 70–75 min. and then plated to determine the effect of the inhibitors on bacterial growth rate. Controls containing saline in place of inhibitor were included, and the initial titre of phage and viable bacteria determined on these. Experiments in which the phage multiplication in the control at 20 min., or the bacterial multiplication after 70 min., was less than fivefold were rejected. The results of a typical experiment are given in Table 1. The degree of inhibition of phage and of bacterial multiplication is expressed as an inhibition index =

$$\frac{\text{Log (final titre in presence of inhibitor/initial titre)}}{\text{Log (final titre in control/initial titre)}}.$$

Complete inhibition, or decrease of the initial titre, thus gives an index of 0; complete absence of inhibition gives an index of 1 (Table 1, cols. 4 and 6). A completely differential inhibitor of phage multiplication should decrease the inhibition index for phage below unity at a concentration that does not affect the index for *E. coli* strain B. However, substances which gave a phage index of 0.5 or less and a bacterial index of 0.9 or more have been regarded as differential inhibitors.

Twelve of the substances tested were not differential inhibitors (Table 2) and four of them were (Table 3). The compounds were tested at concentrations giving phage indexes from 0 to 1 in all cases except sodium cacodylate, which did not inhibit at 0.1 M, and sodium fluoroacetate, which did not inhibit at 100 µg./ml. (1×10^{-6} M), the highest concentration tested. With the same exceptions, all compounds were tested at concentrations giving bacterial inhibition indexes from less than 0.5 to 1. These results modify some of those presented by Czekalowski & Dolby (1949).

Effect of inhibitors on free phage and on adsorption of phage

Dilutions of phage in GTP broth containing about 5×10^7 T2r⁺ particles/ml. were incubated with each of the four active inhibitors for 4 hr. at 37° to determine whether they had any action on the phage alone (Table 4). Arsenite,

borate and cyanide were without effect at concentrations higher than those used in inhibition experiments. Proflavine inactivated coliphage T2r⁺ at 5×10^{-5} M. This is a lower inhibitory concentration than that recorded by Foster (1948), but is in accord with the results of Hedén (1951). In other experiments there was considerable destruction of coliphage T2r⁺ even at

Table 1. *Effect of various concentrations of compounds on the multiplication of bacteriophage and bacteria*

Compound	Final concentration ($M \times 10^{-3}$)	Bacteriophage		Bacteria	
		Titre after 20 min. ($\times 10^6$)	Inhibition index*	Viable count after 75 min. ($\times 10^6$)	Inhibition index*
Saline	—	297	—	353	—
Proflavine	0.001	248	0.91	366	1.02
	0.0025	143	0.63	345	0.99
	0.005	63	0.21	336	0.97
	0.01	8	0	271	0.85
	0.02	4	0	213	0.70
Zinc acetate	0.05	353	1.09	390	1.06
	0.10	365	1.11	370	1.03
	0.20	241	0.89	355	1.00
	0.50	14	0	196	0.66

Ten ml. log phase culture of *Escherichia coli* strain B in GTP broth added to duplicate tubes of 1 ml. inhibitor at -10 min. Viable count on saline control at -2 min.: 64×10^6 organisms/ml. 0.1 ml. T2r⁺ dilution added to one of each pair of tubes at 0 min. to give phage concentration: 43×10^6 particles/ml.

$$* \text{ Inhibition index} = \frac{\text{Log (final titre in presence of inhibitor/initial titre)}}{\text{Log (final titre in control/initial titre)}}$$

5×10^{-6} M-proflavine and some evidence that destruction was less when the mixture was incubated in the dark. This apparent photo-inactivation may be responsible for the discrepancies between the results of different workers. The adsorption of phage to *Escherichia coli* strain B was not affected by the presence of arsenite (2×10^{-3} M), borate (7×10^{-3} M), cyanide (0.8×10^{-3} M), or proflavine (0.01×10^{-3} M).

THE COURSE OF PHAGE MULTIPLICATION IN THE PRESENCE OF INHIBITORS

The effects of arsenite, borate and cyanide on phage multiplication were studied in greater detail by single-step growth curves in the presence of these inhibitors. For this purpose stable suspensions of phage + bacteria were prepared in two ways:

(a) By the method of Benzer (1952), in which phage is adsorbed on to washed bacteria in a non-nutrient medium. A log phase culture in GTP broth of *Escherichia coli* strain B (1×10^8 organisms/ml.) was centrifuged at 3000 r.p.m. for 20 min., washed twice on the centrifuge with phosphate saline,

Table 2. *Inhibition indexes of compounds which did not show differential inhibition*

Compound	Final concentration ($M \times 10^{-3}$)	No. of experiments	Average inhibition index	
			Bacteriophage	Bacteria
Azide, Na	0.9	2	0	0.10
	0.6	3	0.15	0.50
	0.4	2	0.63	0.96
	0.3	4	0.90	1.03
	0.2	3	0.98	0.96
Cacodylate, Na	100	1	0.98	1.14
4:6-Dimethoxytoluquinone	2.5	1	0	0.30
	1.0	2	0	0.64
	0.5	1	1.02	0.86
	0.25	1	c. 1.6	1.08
2:4-Dinitrophenol	1.0	1	0	0.46
	0.3	2	0.5	0.73
	0.2	3	0.44	0.91
	0.15	2	1.00	0.90
Fluoride, Na	50	1	0	0.34
	25	1	0	0.61
	12.5	1	0.79	0.94
	6.2	1	1.02	1.00
Fluoroacetate, Na	0.001	3	1.05	0.96
8-Hydroxyquinoline	0.05	1	0	0.66
	0.03	2	0.28	0.83
	0.02	2	0.79	0.91
	0.015	2	0.89	1.00
	0.010	3	0.88	1.05
	0.0075	2	0.96	1.04
Monoiodoacetate, Na	0.10	1	0	0.24
	0.05	2	0.03	0.77
	0.025	2	0.61	0.93
	0.01	2	0.98	0.88
	0.005	1	1.01	0.99
Sulphanilamide	20	1	0	0.09
	15	2	0.53	0.90
	10	2	0.84	1.10
	5	1	1.00	0.96
Uranyl acetate	4.0	1	0	0
	2.0	2	0.45	0.67
	1.0	2	0.81	0.84
	0.5	2	0.99	1.04
Urethane	300	1	0	0.08
	150	1	0.19	0.80
	75	1	0.5	0.97
Zinc acetate	0.5	1	0	0.66
	0.4	1	0.14	0.72
	0.2	1	0.89	1.00
	0.1	2	1.00	1.01

resuspended in phosphate saline at about 1×10^9 organisms/ml. and aerated for 1 hr. to remove intracellular nutrients. A viable count was made and T2r⁺ particles added to a multiplicity of infection of 3-5. After adsorption at 37° for 10 min. the bacteria with adsorbed phage were centrifuged,

Table 3. *Inhibition indexes of compounds showing differential inhibition*

Compound	Final concentration (M $\times 10^{-3}$)	No. of experiments	Average inhibition index	
			Bacteriophage	Bacteria
Arsenite, K or Na	1.0	3	0	0.68 (0.66-0.72)
	0.5	3	0.38 (0.23-0.58)	0.98 (0.91-1.03)
	0.25	3	0.87 (0.73-1.08)	0.94 (0.88-0.96)
	0.10	2	1.04 (1.04)	1.01 (1.00-1.01)
Borate, Na	5.0	3	0	0.78 (0.69-0.91)
	4.0	3	0.07 (0-0.2)	0.94 (0.92-1.01)
	3.0	4	0.24 (0.09-0.33)	1.06 (1.02-1.12)
	2.0	4	0.60 (0.49-0.84)	1.08 (0.99-1.14)
	1.0	2	0.73 (0.61-0.84)	1.07 (1.05-1.08)
	0.5	1	0.83	1.16
	0.25	1	0.91	1.21
Cyanide, K	0.4	3	0	0.83 (0.78-0.85)
	0.2	3	0.30 (0.0-0.50)	0.95 (0.94-0.96)
	0.1	3	0.50 (0.41-0.68)	0.99 (0.92-1.05)
	0.05	2	0.81 (0.76-0.85)	1.00 (1.00)
Proflavine	0.01	3	0	0.90 (0.85-0.95)
	0.005	3	0.07 (0-0.21)	0.95 (0.84-1.03)
	0.0025	2	0.39 (0.15-0.63)	1.02 (0.99-1.04)
	0.001	2	0.95 (0.91-0.98)	1.01 (0.99-1.02)

Figures in parentheses show the range in individual experiments.

Table 4. *Effect of inhibitors on phage T2r⁺ alone*

Inhibitor	Titre of phage after 4 hr. at 37° ($\times 10^6$)
Saline control	44.5
Arsenite, 2×10^{-3} M	46.3
Borate, 7×10^{-3} M	41.0
Cyanide, 0.8×10^{-3} M	41.0
Proflavine, 0.05×10^{-3} M	11.1

Ten ml. phage, diluted in GTP broth, added to 1 ml. inhibitor in saline, to give the final concentration shown in Table. Incubated 4 hr. at 37°, then diluted and assayed.

washed once on the centrifuge with phosphate saline to remove free phage, and resuspended in phosphate saline at $5-10 \times 10^7$ bacteria/ml. Such suspensions, and dilutions of them in phosphate saline, were stable for at least 1 hr. at 30°, but developed normally on addition of nutrient medium and had a latent period of normal length (28 min. at 30°). In one experiment portions of a dilute suspension in phosphate saline were added 1, 15, 30 and 60 min. after preparation to GTP broth to give a count of 1.1×10^3 plaques/ml. After

100 min. incubation at 30° the respective titres were 139, 119, 146 and 134×10^3 plaques/ml. Burst sizes of over 100 were, however, unusual, the normal range being 50–100. This variation might have been due to variation in the composition of the medium and in the conditions of centrifugation and aeration. Attempts to improve reproducibility by substituting for broth the defined medium of Friedlein (1928) either alone, +0.1 % Tryptose, or +yeast extract, were unsuccessful.

(b) In later experiments a method was used which proved simpler and gave larger burst sizes. To a log phase culture of *Escherichia coli* strain B (1×10^8 organisms/ml.) in GTP broth at 37° were added cyanide or arsenite to a concentration of 1×10^{-3} M and phage T2r⁺ to give a final concentration of 1×10^5 particles/ml., and the culture was incubated for 10 min. at 37°. More than 98 % of the phage was adsorbed, as measured by the supernatant titre after centrifuging, but its development was prevented by the cyanide or arsenite. On dilution into GTP broth to bring the concentration of cyanide or arsenite below its effective level the phage developed normally and gave burst sizes over 100.

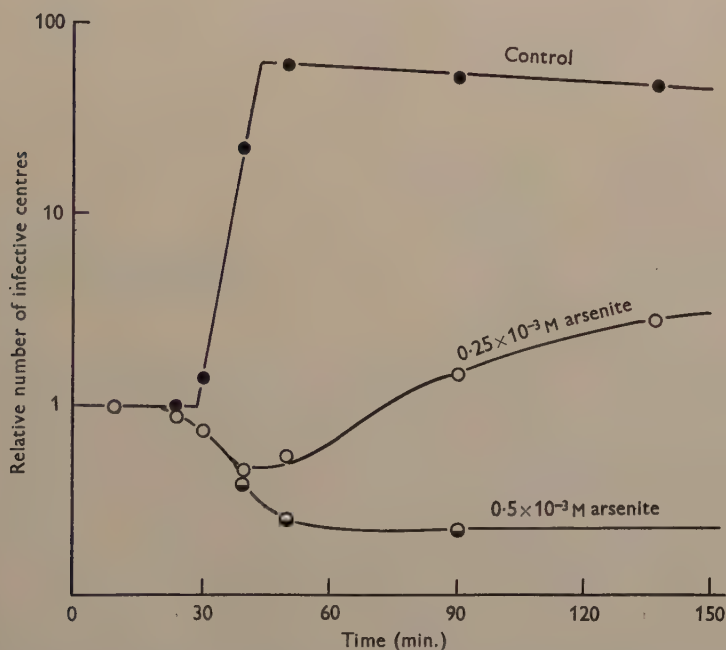
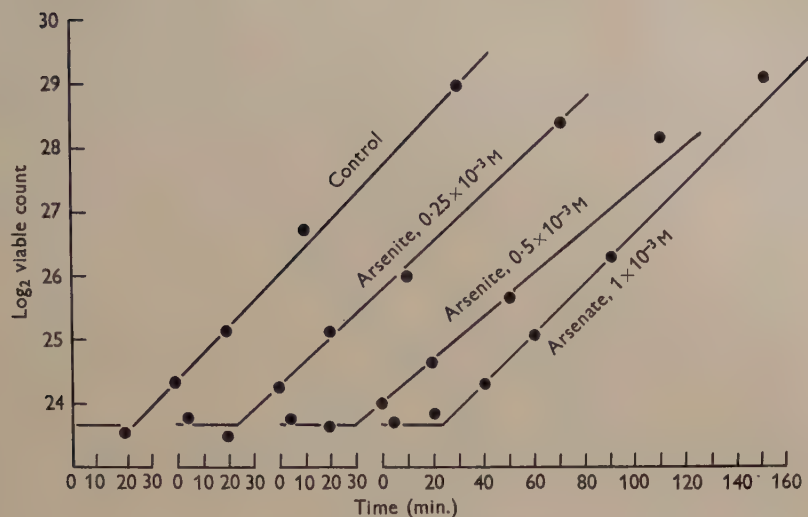
Both types of suspension were added to GTP broth at 30°, containing different concentrations of inhibitor, to give an initial count of 10^3 plaques/ml. and the increase in titre followed as usual in single step experiments. The lower temperature of incubation (30°) gave slower multiplication and more time for manipulation, the latent period being increased from 19 min. at 37° to 28 min. at 30°.

Multiplication of *Escherichia coli* strain B was followed by viable count after adding a washed aerated suspension of bacteria in phosphate saline to GTP broth + inhibitor. The initial titre was $10\text{--}30 \times 10^6$ organisms/ml. The effect of inhibitors is expressed as the ratio of the uncorrected mean generation time (MGT) of the inhibited and control cultures:

$$\frac{\text{MGT of culture + inhibitor}}{\text{MGT of control culture}}.$$

Arsenite. Fig. 1 shows that 0.25 and 0.5×10^{-3} M-arsenite had little effect on the growth of *Escherichia coli* strain B their MGT ratios being 1.08 and 1.20 respectively. A higher concentration of arsenate (1×10^{-3} M) was not inhibitory at all (MGT ratio = 1.02). But the same concentrations of arsenite greatly decreased phage yield in single step experiments (Fig. 2). The yield was never more than sevenfold in the presence of 0.25×10^{-3} M-arsenite and 0.5×10^{-3} M-arsenite suppressed multiplication completely. Arsenite thus appears a more effective inhibitor in these experiments than in the preliminary survey, in which 0.5×10^{-3} M-arsenite did not cause complete inhibition. The concentration of bacteria and phage was many thousand times higher in the early experiments and this probably explains the difference, since the number of inhibitor molecules/bacterium was higher in the single step experiments. Both methods of preparing the phage + bacteria suspensions gave similar results.

Cyanide at 0.2 and 0.4×10^{-3} M had effects similar to those of arsenite



(Figs. 3, 4). Doermann (1952) has already shown that at the higher concentration of cyanide ($0.01M$) used in his method for studying intracellular phage, there is a loss of infectious centres during the first half of the latent period but the bacteria are not lysed by the cyanide; lysis occurs only when the cyanide is added near the end of the latent period. Cyanide thus has two separate effects, inhibition of phage multiplication when it is added early in

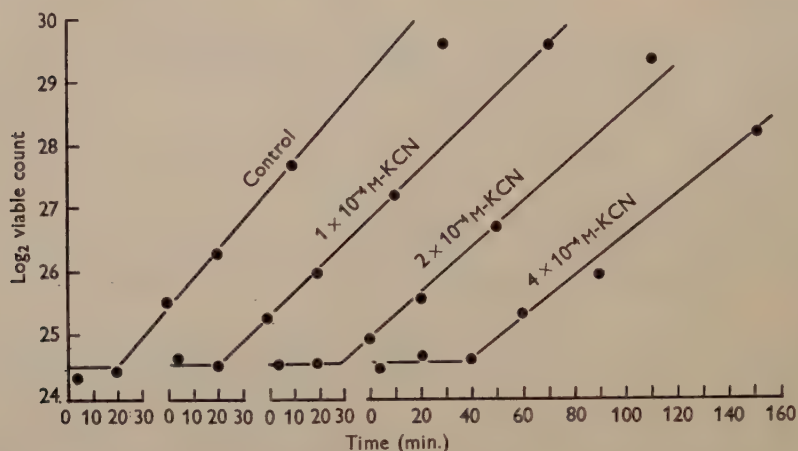


Fig. 3. Effect of cyanide on growth of *Escherichia coli* strain B, measured by viable count. Initial titre: 24×10^6 viable organisms/ml. 37° .

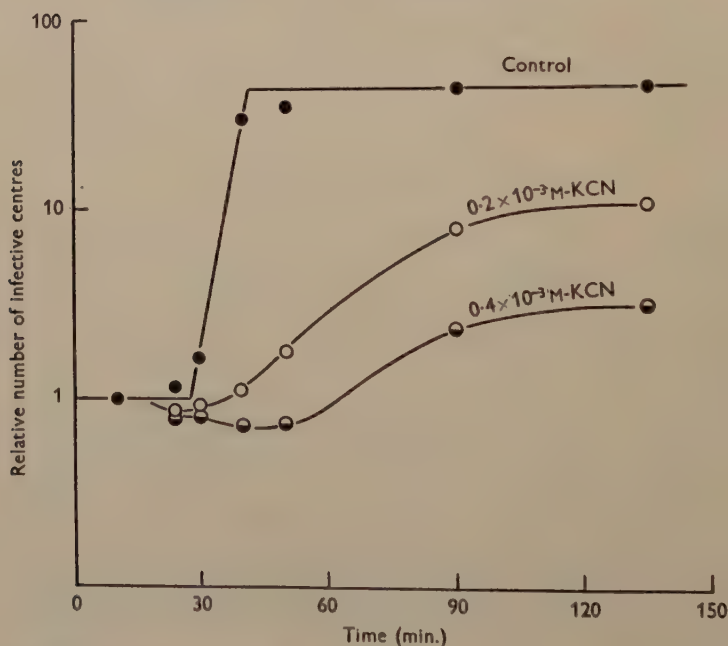


Fig. 4. Effect of cyanide on growth of phage $T2r^+$ in single step experiment. 30° .

the latent period, and lysis when it is added after some intracellular phage particles are completed. Only the inhibitory effect has been studied in the present experiments.

Borate at 2 , 4 and $8 \times 10^{-3}M$ gave average MGT ratios for growth of the bacteria of 1.04 , 1.08 and 1.14 respectively, with no increase in the length of the lag phase. Experiments in which the phage + bacteria suspension was prepared by washing and aeration of the bacteria gave no evidence that these concentrations of borate inhibited phage development; single step growth curves with and without inhibitor were identical. But when the suspensions

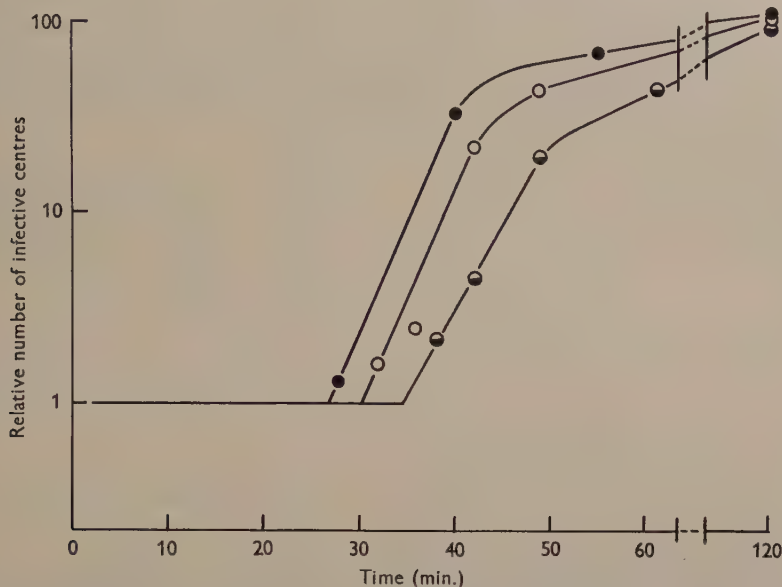


Fig. 5. Multiplication of phage T2r⁺ in single step experiment in presence of borate. Suspension of phage + bacteria, arrested by $1 \times 10^{-3}M$ -cyanide, diluted 1/100 into GTP broth + borate. 30°. ●—●, Control; ○—○, $4 \times 10^{-3}M$ -borate; ◐—◐, $8 \times 10^{-3}M$ -borate.

of phage + bacteria whose development had been arrested by cyanide or arsenite were diluted into GTP broth containing borate inhibition did occur. The latent period was increased from 28 min. to 30–31 min. by $2 \times 10^{-3}M$ -borate, to 31–33 min. by $4 \times 10^{-3}M$, and to 36 min. by $8 \times 10^{-3}M$, but the final titres were almost identical with that of the control, though some decrease occurred in a few experiments. When a suspension of phage + bacteria inhibited by cyanide was centrifuged down, resuspended in fresh GTP broth + cyanide, and then diluted into GTP broth + borate, the usual increase in latent period did not take place. The process of centrifugation and resuspension had evidently annulled the inhibition. This provides an explanation for the fact that suspensions of phage + bacteria prepared by washing and aeration were not inhibited by borate. The results of these experiments justify the assumption made earlier (see SCREENING TESTS) that the length of the latent period might prove a more sensitive indicator of inhibition than decrease in final titre.

Effect of time of addition of inhibitor on phage production

In experiments to determine how late in the latent period the addition of inhibitors remained effective, washed suspensions of phage+bacteria in phosphate saline were added as usual to GTP broth to start phage multiplication; further dilutions were made at intervals into broth+inhibitor at 30°, to give $1-3 \times 10^3$ plaques/ml. and the single step growth curve followed. Inhibition by arsenite occurred when the dilution into arsenite was made as late as 15 min. after the beginning of the latent period (Fig. 6), and other experiments showed complete inhibition when the dilution was made at 15 min. and partial inhibition even when it was made at 22 min. Results with cyanide were similar, although the inhibition at 10 min. was less complete and there was none at 20 min., a result to be expected from the experience of Doermann (1952) in his cyanide lysis experiments.

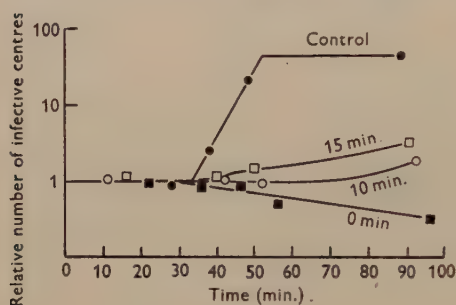


Fig. 6

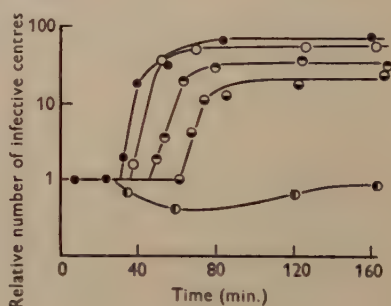


Fig. 7

Fig. 6. Effect of addition of 1×10^{-3} M-arsenite at different times during latent period to phage T2r+ in single step experiment. 30°.

Fig. 7. Effect of removal of 0.5×10^{-3} M-arsenite at different times during latent period of phage T2r+ in single step experiment. 30°. ●—●, Control without arsenite; ○—○, arsenite 0-10 min.; ●—●, arsenite 0-20 min.; ●—●, arsenite 0-30 min.; ●—●, arsenite present throughout.

When arsenite present at the beginning of the experiment was diluted out at different times during the latent period, its effect was to delay the end of the latent period by approximately the time the inhibitor had been present (Fig. 7). In this it resembles most other phage inhibitors.

DISCUSSION

The results described in this paper show that an examination of the effect of enzyme inhibitors on phage multiplication will readily reveal some which inhibit phage differentially. Spizizen (1943) made a similar approach by testing the effect of a number of enzyme inhibitors on the multiplication of washed cells of *Escherichia coli* strain B₁+phage P₁ in phosphate buffer+glycine anhydride (0.87×10^{-3} M) or in broth, finding complete inhibition by NaCN (2×10^{-3} M), moniodoacetic acid (0.5×10^{-3} M), sodium arsenite (0.6×10^{-3} M), 2:4-dinitrophenol (0.5×10^{-3} M) or *p*-amino-phenol

(1×10^{-3} M). Spizizen's conditions, however, differed from those in the present work since the bacteria were unable to multiply in the glycine anhydride, and he did not test the growth of the bacteria in broth which contained the same concentration of inhibitor. The present experiments did not reveal any differential effects of monoiodoacetic acid or 2:4-dinitrophenol, but did confirm those of cyanide and arsenite. The ratio concentration of inhibitor required to suppress growth of phage to concentration required to inhibit bacteria is not as great with arsenite, borate and cyanide as with some other inhibitors, but the effect was clear and reproducible.

Arsenite and cyanide inhibit respiratory and fermentative processes, arsenite by combining with SH-containing enzymes and cyanide by forming complexes with heavy metals. *Escherichia coli* strain B possesses two routes of carbohydrate breakdown, the normal fermentative one and an oxidative pathway via phosphogluconic acid and pentose phosphates. The two are in an equilibrium which may be altered by varying conditions, so that inhibition of one by cyanide or arsenite leads to increased use of the other pathway and a decrease in the effect of the inhibitor. When the cells are infected with coliphage T2r⁺, however, there is a shift in glucose metabolism from the oxidative to the anaerobic pathway (Cohen, 1951; Cohen & Roth, 1953), and the simplest explanation of the present results is that the equilibrium is disrupted by phage infection and that arsenite and cyanide inhibit the anaerobic pathway specifically.

Borate was tested for inhibitory activity because it is known to combine with ribose-5-phosphate and ribulose-5-phosphate, both products of the oxidative respiratory pathway of *Escherichia coli* (Horecker, Smyrniotis & Seegmiller, 1951). It is clear that its mode of action differs from that of cyanide and arsenite. Since the change from the oxidative to the anaerobic pathway referred to above occurs after infection with phage, borate should be effective as an inhibitor only so long as the oxidative pathway is still in use and it should not alter the phage yield. The lengthened latent period and unchanged phage yield found are thus consistent with the hypothesis that borate acts on pentose phosphate.

It is a pleasure to acknowledge a gift of sodium fluoroacetate from Professor Sir Rudolf Peters.

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The Isolation and Characteristics of an Oxalate-decomposing Organism

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SUMMARY: The isolation from soil of three strains of oxalate-decomposing organisms different from other known oxalate-decomposing organisms is described; only one strain was studied in detail. This organism grew in concentrations of potassium oxalate up to 2% (w/v) with an optimum of *c.* 1% (w/v). Growth was preceded by a lag period of *c.* 18 hr. which was not decreased by addition of yeast autolysate, bicarbonate or formate. Of several other substrates tested only glycolate, DL-lactate, pyruvate, DL-malate and succinate supported growth. Washed cell suspensions oxidized oxalate and the gas exchanges observed corresponded with an assimilation of some of the oxalate used. The oxidizing powers of washed cells varied somewhat with the carbon source used for growth.

Very few bacteria have been reported as able to utilize oxalate as the sole source of carbon and energy. The only bacteria which have been isolated in pure culture and shown to decompose oxalate are: *Bacillus extorquens* (Bassalik, 1913); *Vibrio oxaliticus* (Bhat & Barker, 1948); *Pseudomonas extorquens* (Janota, 1950); a species of *Proactinomyces* (Müller, 1950); *Pseudomonas oxaliticus* (Khambata & Bhat, 1953). It has been known for a long time that certain plants (e.g. rhubarb) contain oxalates. Oxalate was one of the first products to be discovered in early studies on the aerobic breakdown of carbohydrate by species of *Aspergillus* and *Penicillium* (Wehmer, 1891), and under certain conditions these fungi also decompose oxalate (Wehmer, 1891; Bach & Fournier, 1935). Although oxalic acid is of wide occurrence in nature relatively little is known of its metabolism; in order to study this process attempts were made to isolate from soil, by the enrichment technique, organisms which used oxalate as sole source of carbon and energy for growth. The present paper deals with the isolation and properties of an aerobic organism of this type. A preliminary account of this work has already been given (Jayasuriya, 1954).

RESULTS

Isolation

For the enrichment cultures a chemically-defined medium (medium A) was used; it had the following composition (mg./100 ml.): KH_2PO_4 , 136; Na_2HPO_4 , 213; $(\text{NH}_4)_2\text{SO}_4$, 50; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$, 0.25; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.25; potassium oxalate, $\text{K}_2\text{C}_2\text{O}_4 \cdot \text{H}_2\text{O}$, 1000. Volumes (10 ml.) of medium A were inoculated with *c.* 5 g. samples of garden soil, one from Pacific Grove, California, and the other from a herbaceous border in Sheffield. Separate enrichment cultures were set up at 25° and 37°

under aerobic and anaerobic conditions. The aerobic enrichment cultures were carried out in 50 ml. Erlenmeyer flasks, each containing 10 ml. medium A. The anaerobic enrichment cultures were set up in 60 ml. bottles, having ground glass stoppers, completely filled with the medium.

Microscopic examination of the cultures after 48 hr. revealed the presence, in all cultures, of a large number of morphological types of organisms with no single one predominating. A loopful of each culture was transferred into fresh medium A. A second series of subcultures were also made in medium A + 0.01 % (v/v) yeast autolysate (Barker & Beck, 1942). Within 48 hr. the 25° aerobic cultures became turbid and titration with 0.1N-KMnO₄ showed that oxalate was being decomposed. There was neither marked growth of organisms nor any appreciable decrease in the permanganate titre in subcultures from the 37° aerobic cultures or in any of the anaerobic cultures.

Organisms from the 25° aerobic cultures were isolated by plating out on a calcium oxalate agar medium similar to that used by Bhat & Barker (1948). This medium contained the components of medium A + 3 % (w/v) washed agar (prepared from Davis New Zealand agar by soaking the agar in distilled water for 24 hr. followed by decanting the water and then repeating the process 3 times) + 0.1 % (v/v) yeast autolysate. Before pouring the plates 10 ml. sterile 0.1M-CaCl₂/100 ml. medium were added to convert part of the oxalate present to calcium oxalate. After incubation for 48 hr. many small colonies appeared. The slow growth of the oxalate-decomposing organisms made their isolation difficult since the colonies tended to be overgrown by contaminants. Slow growth was possibly due to the relative inaccessibility of the carbon source when present as the insoluble calcium oxalate. However, the use of this medium greatly facilitated the recognition of oxalate-decomposing organisms, the colonies of which were surrounded by a clear zone. The organisms picked from these colonies were purified by several successive platings on this solid medium and maintained by fortnightly transfers on aerobic slants.

To confirm that these isolates utilized oxalate, medium A was inoculated from a single colony and the disappearance of oxalate shown by removing samples under aseptic conditions at suitable intervals and determining the oxalic acid content by titration with permanganate. These isolates also grow on silica gel plates prepared according to Meiklejohn (1950) and containing the constituents of medium A.

Three strains of oxalate-decomposing organisms were thus isolated; two from Pacific Grove soil and designated OD1 and OD2; one from Sheffield soil and designated OD3. Organism OD2 was isolated from the second series of subcultures in the presence of yeast autolysate. All three strains were isolated from the 25° aerobic enrichment cultures; no oxalate-decomposing organisms were isolated from either the 37° aerobic enrichment cultures or from any of the anaerobic cultures. The three strains isolated are morphologically similar; the work described below was carried out entirely with the organism OD1.

Organism OD1; morphology and biological properties

Organism OD1 is a short rod of mean dimensions $0.5 \times 1.5 \mu$. It does not stain well by the usual procedures; the only method which gave good preparations was negative staining with nigrosin, but it appears to be Gram-negative. It is actively motile, especially in young cultures. Flagella staining by the method of Bailey (1929), as modified by Fisher & Conn (1942), revealed a single polar flagellum. Neither spores nor capsules were observed. Long filamentous forms were seen occasionally, especially in old cultures.

Pin-point colonies appeared between 24 and 48 hr. on nutrient agar, on 10% (v/v) yeast autolysate agar and on calcium oxalate agar; no colonies were visible up to 24 hr. on any of these media. After 72 hr. the colonies were about 1 mm. diam.; on further incubation the colonies on nutrient agar and on yeast autolysate agar grew to *c.* 2 mm. diam. while the colonies on oxalate agar grew to *c.* 1.5 mm. diam. within about 7 days. All colonies were smooth, raised, opaque and had entire edges. These observations held at 25° and 30°. The organism failed to grow on the above media at 37° and 45°.

In nutrient broth and 10% (v/v) yeast autolysate the organism formed a thin pellicle in 24 hr. at 25° and 30°. This was followed by a heavy turbidity throughout the medium, and at the end of growth there was a heavy deposit on the bottom of the culture vessel. In liquid oxalate medium A, however, only a slight turbidity developed. The organism does not produce any water-soluble pigments, but is itself coloured yellow when grown in nutrient broth or in 10% (v/v) yeast autolysate. The organism does not produce indole; methyl red and Voges-Proskauer tests were negative. Litmus milk was turned slightly alkaline. Catalase is present. Neither acid nor gas was produced in glucose, mannose, sucrose, maltose, lactose, starch or dextrin.

In the presence of 1% (w/v) potassium oxalate as source of carbon and energy, organism OD1 grew with diammonium hydrogen phosphate, ammonium nitrate, sodium nitrate, glycine, L-asparagine or peptone as nitrogen source. Any of these substances at 0.5 g./l. can replace ammonium sulphate in medium A.

A wide variety of carbon compounds was tested as growth substrates for organism OD1 by incorporating them into medium A at 0.1 and 1.0% (w/v) in place of potassium oxalate. Glycollate, DL-lactate, pyruvate, DL-malate and succinate (as sodium salts) were utilized. Organism OD1 also grew on 10% (v/v) yeast autolysate. There was a considerable lag period when media containing these substrates were inoculated with oxalate-grown cells (Table 1); this was, however, shortened by successive subculture in the appropriate medium. However, once growth had started, it was usually complete within 24 hr., as is the case with oxalate as substrate.

Methanol, formaldehyde, formate, ethanol, acetaldehyde, acetate, ethylene-glycol, glycine, glycerol, malonate, propionate, *n*-butyrate, citrate, glucose, mannose, sucrose, maltose, lactose, starch and dextrin were not utilized by organism OD1 in place of oxalate.

Classification of organism OD1

Organism OD1 has not yet been compared directly with other oxalate-decomposing organisms, but from the published descriptions of the latter it would appear to be a different organism; the relevant properties are set out in Table 2. Organism OD1 differs: from *Bacillus extorquens* which is a larger

Table 1. Lags before growth of organism OD1 started on various substrates

The substrate was incorporated into the medium A in place of potassium oxalate. The media were inoculated with oxalate-grown cells.

Substrate	Lag (days)	Total growth (mg. dry wt./l.)
Oxalate (1 %, w/v)	1	66
Glycollate (1 %, w/v)	2	41
DL-Lactate (1 %, w/v)	7	300
Pyruvate (1 %, w/v)	7	—
DL-Malate (1 %, w/v)	7	350
Succinate (1 %, w/v)	14	60
Yeast autolysate (10 %, v/v)	4	300

Table 2. Comparison of the properties of organism OD1 and other known oxalate-decomposing organisms

All four organisms are motile.

	<i>Bacillus extorquens</i>	<i>Vibrio oxaliticus</i>	<i>Pseudomonas oxalaticus</i>	Organism OD1
Dimensions (μ) ...	1.5 \times 3.0	0.4 \times 1.3	0.3 to 0.4 \times 0.9 to 1.5	0.5 \times 1.5
Flagella ...	Single polar	Single polar	1-3 polar	Single polar
Water soluble pigment...	Blood red	—	—	—
Utilization of C sources				
Oxalate	+	+	+	+
Formate	+	+	+	—
Acetate	.	+	+	—
DL-Lactate	.	—	+	+
Citrate	.	—	+	—
DL-Malate	.	—	.	+
Succinate	.	—	.	+

. = Not reported.

organism and produces a blood-red water-soluble pigment; from *Vibrio oxaliticus* which is morphologically different and is able to use acetate and formate but not DL-lactate, DL-malate or succinate; from *Pseudomonas oxalaticus* which can use acetate, formate and citrate. However, two strains (Ox₄ and Ox₆) of *Pseudomonas oxalaticus* (Khambata & Bhat, 1953) used neither citrate nor formate but unlike OD1 where able to use acetate. Organism OD1 also differs from *P. oxalaticus* in being unable to grow at 37°.

The morphology and biological properties of organism OD1 place it in the genus *Pseudomonas*, but it does not resemble any of the species of *Pseudomonas* described in *Bergey's Manual* (1948).

Features of growth of organism OD1 on oxalate

The optimum conditions for growth in liquid medium with oxalate as sole source of carbon and energy were investigated. The galvanometer reading given by the E.E.L. photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, Essex) using filter No. 404, was taken as a measure of growth. As these experiments were for the purpose of comparison, the instrument was not calibrated against dry weight and the values, which express total growth, are given in terms of E.E.L. galvanometer readings.

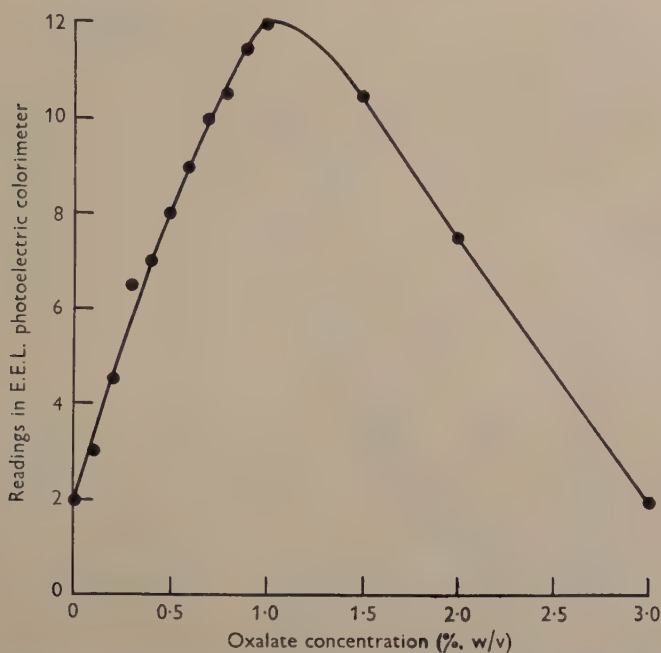


Fig. 1. The effect of the concentration of oxalate on the growth of organism OD1. Oxalate was incorporated into medium A at the concentrations indicated in the figure. After 72 hr. incubation at 25° the total growth was measured by the E.E.L. photoelectric colorimeter using filter no. 404.

Organism OD1 tolerated oxalate in concentrations up to 2% (w/v) with an optimal value of *c.* 1% (w/v) in the mineral medium A (Fig. 1). In concentrations of over 2% (w/v) there was little or no growth. Added growth factors were not required and yeast autolysate when incorporated into medium A at concentrations between 0.0001 and 5% (v/v) did not stimulate growth within 48 hr. Increasing the concentration of inorganic salts up to 15 times the strength used in medium A did not improve growth; there was hardly any growth in the absence of inorganic salts and very high concentrations of salts inhibited growth. Growth occurred when the initial pH value of the medium was in the range 6.5–9.0; optimum 7.0–8.0. The following measurements were

made on samples removed from cultures of organism OD1 during growth in liquid oxalate medium: (a) the optical density of the culture by the Hilger Spekker absorptiometer using the neutral grey Ilford filter no. H508; (b) the nitrogen content of the centrifuged cells by the micro-Kjeldahl method of Chibnall, Rees & Williams (1943), the ammonia being distilled in the apparatus of Markham (1942) and trapped in 1 ml. of the boric acid reagent of Conway & O'Malley (1942); (c) the amount of oxalate which disappeared from the medium as measured by titration with permanganate; (d) the pH value of the culture with the glass electrode.

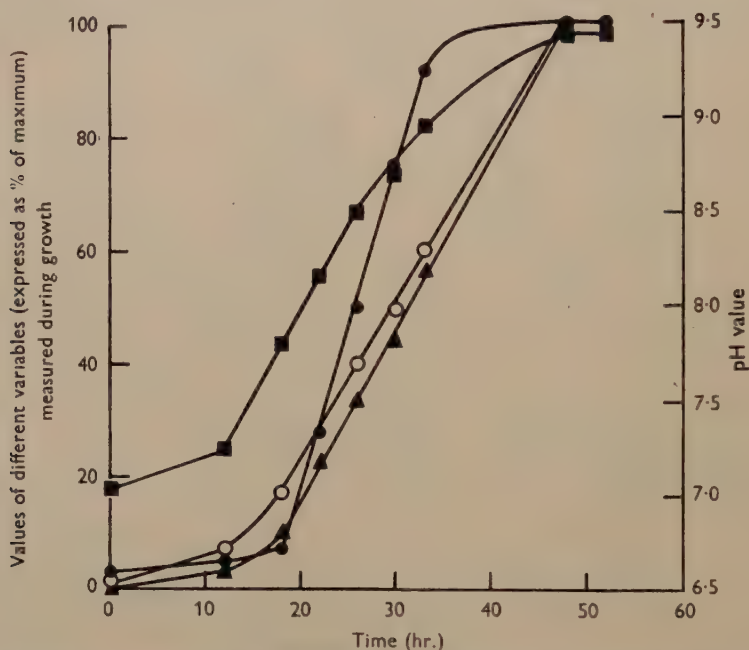


Fig. 2. Growth of organism OD1 on liquid oxalate medium A. Changes measured in the following ways and expressed as a percentage of the maximum values were made during growth: (a) optical density in the Hilger-Spekker absorptiometer using filter no. H508 (●—●); (b) nitrogen content of the cells (○—○); (c) the disappearance of oxalate (▲—▲). The pH value of the culture was also measured at intervals (■—■).

There was a prolonged lag phase of about 18 hr. in each case. This could not be shortened either by using heavier inocula or by reinforcing the medium with sodium bicarbonate \pm sodium formate, at a concentration of 0.1 % (w/v). These substances were tested on the assumption that during the oxidation of oxalate, carbon dioxide and formate might be among the first oxidation products and that a lack of these might be the cause of the lag. The formation of cell nitrogen and oxalate utilization paralleled each other, whereas the maximum optical density was reached before all the oxalate was utilized. During growth the pH value of the medium increased from 7.0 to 9.5 (Fig. 2).

Oxidation of oxalate by washed suspensions of organism OD 1

Organism OD 1 was grown at 25° in liquid oxalate medium A and distributed in 300 ml. portions in penicillin flasks (180 mm. diam., 100 mm. deep, James A. Jobling and Co. Ltd., Sunderland, England). Each penicillin flask was inoculated with 2 ml. of a 30 hr. culture of organism OD 1 grown in the same medium. After 40 hr., and whilst the organism was in the logarithmic phase of growth, the cells were harvested by centrifuging at 2500 g for 15 min. The cells were washed twice with distilled water and suspended in distilled water

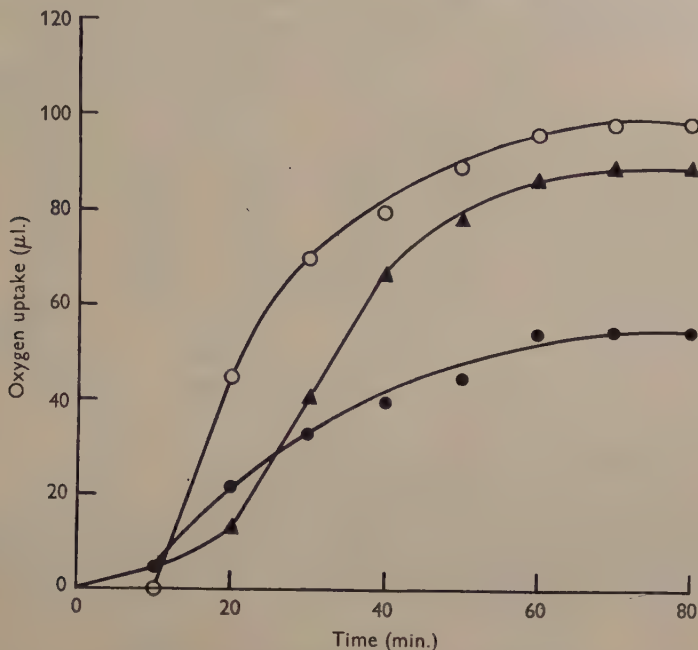


Fig. 3. Oxidation of oxalate by washed suspensions of organism OD 1. Manometer cups contained: main compartment, 1.0 ml. cell suspension (10 mg. dry wt./ml.), 0.5 ml. 0.05 M-triethanolamine buffer (pH 8.0) and inhibitor where indicated; side bulb, 0.1 ml. 0.1 M-oxalate (10 μ mole); centre well, 0.2 ml. 10% KOH. Total volume of contents, 2.2 ml. Results are corrected for endogenous oxygen uptake. Oxalate alone (●—●); oxalate + 0.001 M-azide (▲—▲); oxalate + 0.0005 M-2:4-dinitrophenol (○—○).

to give 10 mg. dry weight cells/ml. Dry weight was determined by drying a sample of the cell suspension for 4 hr. at 105°. Subsequently, dry weights were estimated by referring to a standard graph which related dry weight (4 hr.; 105°) to optical density as measured in the Hilger Spekker absorptiometer with the neutral grey Ilford filter no. H 508.

The oxidation of oxalate by washed suspensions of organism OD 1 was tested manometrically in 0.0125 M-triethanolamine buffer (pH 8.0) at 25°. Gas exchanges were measured by the first method of Dickens & Šimer (1930) as modified by Elsdén & Lewis (1953). The oxidation of oxalate was preceded by a lag period of about 10 min. (Fig. 3). The oxygen uptake was always 60 %

of that required for the complete oxidation of oxalate to CO_2 . In the presence of 0.001 M-sodium azide or 0.0005 M-2:4-dinitrophenol, known to be inhibitors of oxidative assimilation (Clifton, 1937), the oxygen uptake was almost the theoretical for complete oxidation and the respiratory quotients approximated to the theoretical value of 4.0 (Table 3).

Table 3. *The effect of azide and 2:4-dinitrophenol on oxidation of oxalate by washed suspensions of organism OD 1*

Manometer cups contained: main compartment, 1.0 ml. cell suspension (10 mg. dry wt./ml.), 0.5 ml. 0.05 M-triethanolamine buffer (pH 8.0) and inhibitor where indicated; side bulb, substrate; centre well, 0.2 ml. 10% KOH. Total volume of contents, 2.2 ml.

Substrate	Cells alone		Cells + 0.001 M-azide		Cells + 0.0005 M 2:4-dinitrophenol	
	O ₂ uptake (μl.)	CO ₂ output (μl.)	O ₂ uptake (μl.)	CO ₂ output (μl.)	O ₂ uptake (μl.)	CO ₂ output (μl.)
(a) None	42	58	42	61	35	45
(b) 10 μmole oxalate	102	369	146	439	135	443
(b) - (a)	60	311	104	378	100	398
Theoretical values*	112	448	112	448	112	448
R.Q. observed	5.18		3.63		3.98	
R.Q. theoretical	4.00		4.00		4.00	

* Theoretical values for complete oxidation of oxalate to CO_2 .

Table 4. *The oxidation of various substrates by oxalate-grown and lactate-grown cells of organism OD 1*

Manometer cups contained: main compartment, 1.0 ml. cell suspension (10 mg. dry wt./ml. in the case of oxalate-grown cells and 15 mg. dry wt./ml. in the case of lactate-grown cells) + 0.5 ml. 0.05 M-triethanolamine buffer (pH 8.0); side bulb, substrate; centre well, 0.2 ml. 10% KOH. Total volume of contents, 2.2 ml.

Respiratory substrate	Q _{O₂}	
	Oxalate-grown cells	Lactate-grown cells
Oxalate	43	18
Glycollate	6	19
Acetate	5	10
Formate	6	6
Pyruvate	14	17
DL-Lactate	11	22
Succinate	10	4
Fumarate	7	3

Oxidation of other substrates by washed suspensions of organism OD 1

The ability to oxidize other substrates was tested; it varied with the carbon source used for growth (Table 4). (The lactate medium was prepared by replacing potassium oxalate in medium A by 1% (w/v) sodium lactate.) The main differences to be observed are that when the organism was grown on lactate the Q_{O_2} (μl. O₂ consumed/mg. dry weight/hr.) on lactate or glycollate was increased two to threefold, whereas the Q_{O_2} on oxalate was decreased to about half that observed with oxalate-grown cells.

DISCUSSION

Oxalate-decomposing organisms have been isolated from a variety of habitats. Thus *Bacillus extorquens* (Bassalik, 1913), *Vibrio oxaliticus* (Bhat & Barker, 1948) and the organism described in this paper were isolated from soil, whereas the oxalate-decomposing *Proactinomyces* sp. (Müller, 1950) was isolated from sheep rumen and *Pseudomonas oxalaticus* (Khambata & Bhat, 1953) from the alimentary tract of an Indian earthworm. Organism OD1 appears to resemble most closely *P. oxalaticus*, but there are certain differences in the biochemical properties of the two organisms; and further OD1 does not stain readily, whereas Khambata & Bhat (1953) do not appear to have experienced any such difficulty with *P. oxalaticus*. On morphological grounds, e.g. its rod-like shape and polar flagellation, OD1 would appear to be a member of the genus *Pseudomonas* but, until such time as a direct comparison has been made with authentic strains of other oxalate-decomposing organisms, it has been decided to refer to this organism as *Pseudomonas* OD strain 1.

Apart from carbon dioxide, oxalate is the most oxidized carbon compound known to be used by bacteria as a sole source of carbon and energy, and it is obvious that any organism which grows upon oxalate must be capable of reducing it to the level of cell material. In this respect, the metabolism of such oxalate-decomposing organisms resembles that of the aerobic autotrophic bacteria which, as a result of the oxidation of inorganic substrates, obtain energy for the reduction of carbon dioxide to cell substance. Consequently a study of the processes involved in the metabolism of oxalate-decomposing bacteria may help towards an understanding of the metabolism of the autotrophic bacteria.

Since oxalate is such a highly oxidized compound the mechanism of its oxidation would seem likely to involve only a few reactions and a possible pathway may be:



Formate is placed in parenthesis since organism OD1 does not grow upon this substance, and it may be that an active form of formate is involved.

Table 3 shows that when oxalate is oxidized by washed suspensions of organism OD1 the amounts of carbon dioxide produced and of oxygen consumed are below those required for complete oxidation. On the other hand, the respiratory quotient is greater than the theoretical, 5.18 as opposed to 4.0. In the presence of either azide or 2:4-dinitrophenol the gaseous exchanges approached the theoretical values for complete oxidation to CO_2 and the respiratory quotients approximated to 4.0. These calculations have been made on the assumption that the amounts of oxygen consumed and carbon dioxide produced by cell suspensions in absence of substrate could be deducted from the appropriate figures obtained in presence of substrate. This would appear to be justified, at least in so far as the figures for oxygen are concerned, since in the presence of both azide and 2:4-dinitrophenol the total oxygen consumed in the presence of substrate was greater than that required for complete oxidation

of the substrate. The fact that the respiratory quotient is greater than theoretical is in agreement with the view that some of the substrate is being reduced to cell material.

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The Metabolism of Malate and certain other Compounds by *Desulphovibrio desulphuricans*

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SUMMARY: *Desulphovibrio desulphuricans* (strain El Agheila Z) oxidized malate, fumarate, succinate, lactate and pyruvate quantitatively to acetate in cultures containing excess sulphate. Polysaccharide accumulated in old cultures.

Cell suspensions harvested from malate media yielded theoretical amounts of sulphide and CO_2 from malate, fumarate or succinate + excess sulphate. Succinate was formed transiently during malate oxidation. Various inorganic sulphur-containing anions, methylene blue or oxygen acted as alternative hydrogen acceptors to sulphate for malate oxidation. In the absence of sulphate, malate was metabolized yielding acetate, CO_2 and succinate in the molar ratio 3 malate \rightarrow 2 succinate + 2CO_2 + acetate; in hydrogen, malate or fumarate were partly reduced to succinate. Malate or fumarate accelerated the reduction of sulphate, but not dithionite, in hydrogen. Suspensions treated with cetyltrimethylammonium bromide formed fumarate and lactate + CO_2 from malate. Fumarate was formed via a reversible fumarase; lactate was formed via a decarboxylase system independent of cozymase, Mn^{++} , cocarboxylase or codecarboxylase.

Cell suspensions harvested from lactate media yielded theoretical amounts of sulphide and CO_2 from lactate or pyruvate + excess sulphate. In the presence of arsenite, pyruvate was formed from lactate; pyruvate formation was demonstrated with another strain.

Suspensions of strain California 43:63 harvested from lactate media reduced fumarate in H_2 quantitatively to succinate; crotonate, maleate or acetylene-dicarboxylate were not reduced; malate was reduced slowly.

Hence the reaction sequence: succinate \rightleftharpoons fumarate \rightleftharpoons malate \rightarrow lactate \rightarrow pyruvate \rightarrow acetate probably takes place in these bacteria; a reversible succinate \rightleftharpoons fumarate system may form a link between sulphate reduction and the oxidation of organic compounds or hydrogen.

Malate was the carbon source originally used by Beijerinck (1895) for the cultivation of sulphate-reducing bacteria, though lactate, introduced by his pupil van Delden (1903), has since been more widely used. There have been relatively few detailed studies of the metabolism of carbon compounds by sulphate-reducing bacteria. Baars (1930) examined the ability of many organic compounds to support growth of the bacteria and showed that acetate was usually an end product of carbon compound oxidation. Senez (1951) showed that pyruvate oxidation was accompanied by greater growth and less sulphide formation than was lactate oxidation, and Postgate (1952*a*) showed that growth in the absence of sulphate could occur in pyruvate media, the 'sulphate-free' metabolism giving CO_2 , acetate and ethanol among its products (Postgate, 1952*b*). Subsequently, Sadana (1954) obtained an extract which converted pyruvate to ethanol and acetyl phosphate from acetone-dried cells of the Hildenborough strain. Millet (1954) obtained an extract from dried cells of a different strain which converted pyruvate to acetyl

phosphate, CO_2 and H_2 . Senez & Leroux-Gilleron (1954) also recorded formation of hydrogen during pyruvate or cysteine metabolism without sulphate. Senez (1954*a*) showed that the relative rates of oxidation of carbon sources including lactate, pyruvate, malate, fumarate and succinate depended on the media from which the cells had been harvested, and that strain Canet 41 did not attack the dicarboxylic acids without previous 'adaptation' to fumarate. The present paper describes mainly the metabolism of malate by a halophilic strain of *Desulphovibrio desulphuricans*, though stages relevant to the metabolism of lactate, succinate, fumarate and pyruvate have been demonstrated during the work. Part of this work has been reported elsewhere (*Chemistry Research* 1952, 1953).

METHODS

Organisms. *Desulphovibrio desulphuricans* strain El Agheila Z (National Collection of Industrial Bacteria, NCIB 8380) was originally a hydrogenase-free halophilic strain isolated from a sulphur-bearing lake in Libya (Adams, Butlin, Hollands & Postgate, 1951). A hydrogenase-containing variant of that strain was used in this work. Strain New Jersey SW8 (NCIB 8315) was a marine halophilic strain kindly provided by Professor R. L. Starkey. These strains were maintained on a malate medium. Strain California 43:63 (NCIB 8364) was a marine halophilic organism kindly provided by Professor C. E. ZoBell; it was maintained on a lactate medium. Strain Hildenborough (NCIB 8304) was a non-halophilic strain used in other work from this laboratory. All were typical motile obligately anaerobic vibrios; the cultures were checked for purity at intervals using the procedure described by Postgate (1953).

Cultivation. Stocks were kept in a freeze-dried condition, but during work on a particular strain it was maintained by weekly subculture at 30° in either (a) the lactate medium C of Butlin, Adams & Thomas (1949) supplemented with NaCl 2.5 % (w/v), or (b) a similar medium containing sodium hydrogen malate 0.5 % (w/v) in place of sodium lactate. Cultures were grown in $6 \times \frac{5}{8}$ in. test tubes with a plug containing 1:1 (v/v) pyrogallol (sat.) and Na_2CO_3 (sat.). For manometric or fermentation experiments, organisms were harvested by centrifugation from 3- to 5-day-old cultures in flasks (100 ml. to 2 l.) using similar media but containing yeast extract ('Difco') 0.4 % (w/v) and sodium sulphide *c.* 5×10^{-3} M (see Grossman & Postgate, 1953) to accelerate growth. Such organisms were washed once in either NaCl 2.5 % (w/v) or saline phosphate buffer (below).

Standardization of suspensions. To avoid errors due to osmotic leakage, cell suspensions were standardized turbidimetrically and related to a calibration curve of a non-halophilic strain (Hildenborough). Dry weights recorded in this paper are thus the dry weights of a suspension of organisms of the Hildenborough strain of equivalent turbidity.

Detection and estimation of organic acids. These were detected by ascending chromatography on Whatman no. 1 filter paper sheets using *n*-butanol/3 N- NH_4OH (1:1) for steam-volatile acids and *n*-butanol/acetic acid/ H_2O (4:1:5),

di-isopropyl ketone/formic acid/H₂O (4:1:5) or amyl alcohol/formic acid/H₂O (5:2:5) for non-volatile acids. The dried papers were sprayed with bromthymol blue or bromocresol green respectively. All chromatograms were run with markers and without special temperature control. The identity of acetic acid in bacterial products was confirmed by Duclaux distillation; it was estimated by titration in CO₂-free air after steam distillation followed by re-distillation with MgSO₄ + HgO (Friedemann, 1938).

Succinic acid was detected by the resorcinol test and by chromatography. It was estimated in bacterial products by extraction into ether, transfer to water and titration after steam distillation to remove acetic acid. The titration residues were concentrated and chromatographed to confirm the absence of contaminating acids.

Pyruvic acid was estimated by the method of Friedemann & Haugen (1943) and identified by descending chromatography of the 2:4-dinitrophenyl-hydrazone in *n*-butanol/2*N*-NH₄OH (1:1). Lactic acid was estimated by Neish's (1950) modification of Barker & Summerson's (1941) method.

Other estimations. Total carbohydrate was determined by the anthrone reaction (Neish, 1950). Carbon compounds volatile in steam at pH 7.0 were estimated by the procedure given by Neish (1950) for ethanol.

Manometry. The conventional Warburg manometer was used for gas exchange measurements. Cadmium chloride (0.25 ml. of 10 %, w/v) on glass wool was normally used in the centre well or in a detachable container to absorb H₂S displaced by the buffer, and the main compartment, after tipping, contained 2.75 ml. of reaction fluid including substrates, and 5–10 mg. dry wt. cells in saline buffer. Saline buffer contained NaCl (2.5 %, w/v) + KH₂PO₄ (0.5 %, w/v) and the pH was adjusted with 2*N*-NaOH. This buffer was supplemented with Na₂HPO₄ (0.4 %, w/v) for studies with dicarboxylic acids to improve its buffering capacity. Manometers were incubated at 37°; their constants were corrected for CO₂ retention by the buffer at pH values > 5.0. Vessels were flushed out with H₂ (British Oxygen Co.) or 'oxygen-free' N₂ (British Oxygen Co.) containing less than 10 v.p.m. O₂. *Q* values represent mm.³ gas exchanged/mg. dry wt. cells/hr.; the most commonly obtained values for a given substrate are quoted, with the highest and lowest values encountered as the range.

Estimation of sulphide. (a) Cultures. The culture was incubated under N₂ in a sealed vessel filled as full as possible so that errors due to H₂S in the gas phase should be minimal. After growth, 2 ml. samples of culture were pipetted immediately into excess I₂ which was back-titrated against standard Na₂S₂O₃ with starch as indicator. Experiments in this laboratory with cells grown in sulphate-free conditions indicated that 1 mg. dry wt. cells absorbed I₂ equivalent to 2.2 μmole sulphide; this correction was applied to all cultures analysed directly for sulphide. (b) Manometric experiments. The CdCl₂ plug was transferred to acid 0.01*N*-I₂ in stoppered tapered tubes and the residual I₂ titrated against 0.001*N*-Na₂S₂O₃ with CCl₄ as internal indicator (see Postgate, 1951).

Methylene-blue reduction times were measured by incubating washed and buffered cell suspensions (*c.* 0.3 mg./ml.) in conventional Thunberg tubes

in vacuo at 30° with 0.1 μ mole methylene blue (British Drug Houses Ltd., 'biological' grade) and *c.* 7.5 μ mole substrate.

Reagents. Analytical grade reagents were used wherever possible. Sodium hydrogen malate (British Drug Houses Ltd.) was used in the malate medium, but L-malic acid (L. Light and Co.) was used in metabolic experiments. Sodium pyruvate and sodium fumarate were prepared in the laboratory from pyruvic and fumaric acids (L. Light and Co.). Cetyltrimethylammonium bromide (CTAB) recrystallized from acetone was kindly provided by Dr V. A. Knivett.

RESULTS

Preliminary observations

Growth of strain El Agheila Z in the malate medium was detectable after 24 hr., and reached its maximum cell density in 4 days. Growth was similar to that with lactate or fumarate, less than that with pyruvate (see Senez, 1951) but greater than that with succinate (Table 1). After very prolonged incubation the cell density apparently declined owing to coagulation of the bacteria,

Table 1. *Growth and acetate formation in cultures of Desulphovibrio desulphuricans (El Agheila Z) with various carbon sources*

Cultures (10 ml.) were inoculated with 0.1 ml. of parent culture and incubated at 30° in N₂ until maximum cell densities or acetate yields were reached.

(A) *Growth.* Media contained 31 μ mole sodium salt of C-source/ml., 15.5 μ mole SO₄²⁻/ml., 0.1 % yeast extract, Na₂S (5 μ mole/ml.) and various salts (see text). Means of quintuplicate readings quoted.

Substrate of inoculum	Substrate of test cultures	Final cell density (μ g. dry wt. cells/ml.)
Malate	DL-Malate	340
Malate	L-Malate	340
Malate	Fumarate	340
Malate	Succinate	120
Lactate	DL-Lactate	320
Lactate	Pyruvate	590
Either	—	25

(B) *Acetate yield.* Media as above except C-sources reduced to 15.5 μ mole/ml. to leave sulphate in excess. Inoculum substrates as above. Means of triplicate readings quoted.

Test substrate	Final acetate conc. (μ mole/ml.)	Incubation required to reach maximum acetate yield (days)
DL-Malate	18	4
Fumarate	17.5	4
Succinate	18	8
DL-Lactate	18.5	4
Pyruvate	18.2	2
—	2.5	2

Note: 'blank' growth and acetate formation are due to oxidizable carbon in the yeast extract.

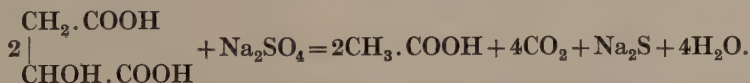
and polysaccharide could be demonstrated in the supernatant medium by the anthrone reaction and the increased viscosity; a 22-day culture gave an anthrone reaction equivalent to $1.0 \mu\text{mole}$ glucose/ml. Similar phenomena were observed in cultures grown with fumarate, succinate or lactate. Senez (1953) mentioned polysaccharide formation in 'semi-autotrophic' cultures of strain Canet 41. Polysaccharide formation by El Agheila Z was not investigated further.

Washed cell suspensions of strain El Agheila Z reduced methylene-blue anaerobically in the presence of malate whether harvested from malate or lactate media, in contrast to New Jersey SW8 (below). Both L-malate and DL-malate were metabolized by cell suspensions in the presence of sulphate, yielding CO_2 at similar initial rates and in similar amounts. Malate was oxidized in the presence of sulphite, thiosulphate, tetrathionate or dithionite (cf. Postgate, 1951); it was also slowly oxidized with oxygen (as a 96% N_2 + 4% O_2 , v/v, mixture) as a hydrogen acceptor ($-Q_{\text{O}_2}$ c. 10); Postgate (1954b) showed reactions of this kind with the Hildenborough strain and demonstrated that the hydrogen/oxygen reaction was cytochrome-linked.

Five other strains of *Desulphovibrio desulphuricans* tested by the methylene-blue procedure showed the presence of a malic dehydrogenase system, even when they had never been grown in a malate medium. A sixth strain, New Jersey SW8, did not show this (Table 5), but after subculture in malate medium it developed the ability to reduce methylene blue with malate; thus its behaviour resembled that of Senez's Canet 41.

Malate oxidation during sulphate reduction

Washed suspensions of El Agheila Z harvested from malate medium oxidized L-malate in the presence of excess sulphate to yield $2 \mu\text{mole}$ $\text{CO}_2/\mu\text{mole}$ malate oxidized (Fig. 1); $0.5 \mu\text{mole}$ S^{2-} was formed (Table 3). No gas change was observed when there was NaOH in the centre well, hence no gas other than CO_2 was formed. Analyses of cultures grown in the malate medium compared with control cultures from which malate was omitted indicated a maximum yield of one μmole acetate/ μmole malate (Table 1). Thus the total yields of CO_2 , sulphide and acetate were consistent with the formal equation:



However, two types of experiment showed that the reaction was more complex than indicated in this equation. First, the equation requires that the acetate:sulphide ratio in cultures at all stages of growth should be 2:1, but analyses of cultures before all the malate had been converted to acetate gave ratios nearer to 3:1. Typical figures were: (a) a 3-day culture contained $7.0 \mu\text{mole/ml}$. acetate and $2.43 \mu\text{mole/ml}$. sulphide (ratio = 3:1.04); (b) 10-day culture contained $15.25 \mu\text{mole/ml}$. acetate and $5.02 \mu\text{mole/ml}$. sulphide (ratio = 3:0.99). Succinate, lactate and fumarate were sometimes detected chromato-

graphically in ether extracts of such cultures. Secondly, CO_2 -evolution curves showed that malate oxidation by cell suspensions at pH 6.1 took place at two distinct rates (Fig. 1). These double-rate curves were responsible for the difficulties reported in establishing CO_2 :malate ratios in earlier stages of this work (*Chemistry Research* 1952, 1953). Malate was oxidized with an initial Q_{CO_2} of *c.* 40 (variance: 30 to 60) and after *c.* $1.3 \mu\text{mole CO}_2/\mu\text{mole malate}$ had been evolved the reaction rate slowed down to a Q_{CO_2} of *c.* 10 (range: 5 to 15). Cells with sulphate but without malate had a Q_{CO_2} of *c.* 2.5 (range: 2 to 3). The lower quotient with malate corresponded to that of a similar cell suspension oxidizing succinate; lactate or pyruvate was usually oxidized faster than succinate but slower than malate, though cell suspensions sometimes did not oxidize lactate or pyruvate at all. The form of the curves suggested accumulation of an intermediate which was then metabolized at a slower rate than malate. The rate suggested that it might be succinate, which was isolated and characterized (see Methods) by interrupting the metabolism of malate at various stages, ether-extracting the acidified supernatant and chromatographing the extract. Succinate appeared soon after the start of malate oxidation but had disappeared by the time CO_2 evolution had fallen to the 'blank' rate.

Malate dismutation without sulphate

Malate was metabolized more slowly by strain El Agheila Z in the absence of sulphate; Q_{CO_2} *c.* 20 (range: 15 to 25). No gas other than CO_2 was formed. When methylene blue was added after CO_2 evolution had ceased, it was decolorized, indicating accumulation of a substance capable of acting as a

Table 2. *Malate metabolism of Desulphovibrio desulphuricans (El Agheila Z) without sulphate*

Acetate and succinate were estimated after fermentation of $30 \mu\text{mole/ml.}$ of malate in N_2 at 37° by cell suspensions (4–10 mg. dry wt./ml.) until manometric controls indicated that all CO_2 evolution had ceased (5–27 hr., depending on suspension density used).

	Succinate ($\mu\text{mole/ml.}$)	Acetate ($\mu\text{mole/ml.}$)	Controls without malate	
			Succinate ($\mu\text{mole/ml.}$)	Acetate ($\mu\text{mole/ml.}$)
	23.2	9.0	—	—
	19.9	10.4	0.7	1.4
	20.6	11.9	2.1	1.8
	19.4	11.7	—	—
Mean	20.8	10.75	1.4	1.6
σ	1.46	1.16	—	—

hydrogen donor in the bacterial metabolism. A similar effect was reported in the dismutation of pyruvate without sulphate (Postgate, 1952*b*). This hydrogen donor was not free hydrogen since only CO_2 was present in the gas phase and, when sulphate was added after malate dismutation had ceased, CO_2 was evolved and sulphide formed.

Examination of the end products of malate dismutation without sulphate

showed the presence of succinate. Manometric experiments showed that 0.62 to 0.81 $\mu\text{mole CO}_2$ was evolved/ μmole malate metabolized (mean of six experiments = 0.716 $\mu\text{mole CO}_2$, $\sigma = 0.0575$); rather high 'blank' values for CO_2 evolution were encountered in these experiments. Fermentation experiments in which cell suspensions were shaken with relatively large quantities of substrate until manometric controls indicated that gas evolution had ceased yielded approximately 2 μmole succinate and 1 μmole acetate from 3 μmole malate (Table 2). No increase in the total polysaccharide content of cells could be demonstrated, but traces were detected of a compound which was volatile in steam at neutral pH and which was oxidized by acid dichromate. It was not identified unequivocally as ethanol, but, calculated as ethanol, it accounted for less than 3% of the malate utilized.

Formation of fumarate

Succinate, CO_2 and acetate were thus the main products of malate dismutation. Attempts were made to demonstrate fumarate formation during the formation of succinate from malate. Acetone-dried cells, cell extracts obtained by grinding with Al_2O_3 , or cells metabolizing malate in air did not accumulate fumarate, but cells treated with cetyltrimethylammonium bromide (CTAB) did so. In a typical experiment, a cell suspension in distilled water of 50 μg dry wt. cells/vessel (1 ml.), treated with CTAB (50 μg /mg. dry wt. cells) and incubated with 125 μmole malate or fumarate in N_2 , formed a mixture of malate and fumarate from either substrate, the products being identified chromatographically.

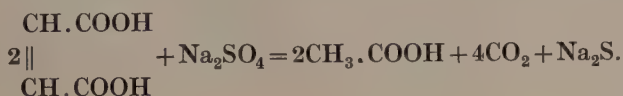
Succinate oxidation

Since succinate was clearly involved in malate metabolism, its oxidation by *El Agheila Z* grown in malate was examined. Succinate was not metabolized in the absence of sulphate, but in its presence cell suspensions slowly yielded 2 $\mu\text{mole CO}_2$ / μmole succinate (Q_{CO_2} c. 10) and 0.75 μmole sulphide (Table 3). Analyses of cultures (Table 1) showed that 1 μmole acetate was formed/ μmole succinate utilized. These observations are consistent with the equation:



Fumarate metabolism

With sulphate. Fumarate was metabolized in a manner similar to malate: a rapid initial Q_{CO_2} of c. 40 was observed and, after c. 1.3 $\mu\text{mole CO}_2$ / μmole fumarate had been evolved, a lower Q_{CO_2} value of c. 10. Sulphide was formed to the extent of 0.5 $\mu\text{mole S}''$ / μmole fumarate oxidized (Table 3). Analyses of cultures grown with excess of sulphate yielded 1 μmole acetic acid/ μmole fumarate (Table 1). These observations agree with the equation



Without sulphate. Cell suspensions without sulphate metabolized fumarate and malate at similar rates—consistent with the presence of fumarase in the cells—though CO_2 yields with fumarate were consistently slightly higher than with malate. Three separate determinations gave 0.78, 0.71 and 0.89 $\mu\text{mole CO}_2/\mu\text{mole fumarate}$ (cf. Table 2); succinate and acetate were detected as end products of the reaction.

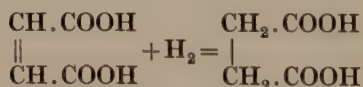
Table 3. *Sulphide yields from Desulphovibrio desulphuricans strain El Agheila Z with sulphate and various carbon sources*

Cell suspensions were shaken in Warburg manometers (N_2 , 37°) and the sulphide formed was determined after CO_2 evolution had reached the 'blank' level. Figures are corrected for blank sulphide yields ($< 0.5 \mu\text{mole S}''$).

Substrate (μmole)	Na_2SO_4 (μmole)	Cell density ($\mu\text{g. dry wt./ml.}$)	Sulphide formed (μmole)	Theoretical sulphide (μmole)
Lactate* 5	5	1.75	2.46	2.5
Pyruvate* 5	5	3.2	1.25	1.25
L-Malate 5	20	4.5	2.65	2.5
Fumarate 5	20	7.6	2.45	2.5
Succinate 5	20	10	3.69	3.75

* Substratum maintained in the lactate medium.

Fumarate reduction. Sisler & ZoBell (1951) reported certain autotrophic marine strains of *Desulphovibrio desulphuricans* which were able to reduce fumarate with hydrogen in place of sulphate; sometimes their strains reduced fumarate faster than sulphate. Since fumarate might be acting as a hydrogen acceptor in the formation of succinate from malate by El Agheila Z, fumarate reduction was investigated further. Strain California 43:63, grown on lactate medium ($-\text{Q}_{\text{H}_2}$ with sulphate *c.* 20) reduced fumarate in hydrogen with a $-\text{Q}_{\text{H}_2}$ of *c.* 37 (range: 47 to 20) and absorbed 1 $\mu\text{mole H}_2/\mu\text{mole fumarate}$ (Fig. 2). Succinate was identified in the reaction products. This suggested a reaction which may be represented:



Malate was reduced very slowly ($-\text{Q}_{\text{H}_2}$ *c.* 16, compared with 'blank' $-\text{Q}_{\text{H}_2}$ of *c.* 13), but otherwise the reaction was highly specific; maleate, crotonate or acetylene dicarboxylate were not reduced, though these acids are structurally similar to fumarate. Fumarate did not replace sulphate for growth of the organism in the autotrophic medium of Butlin & Adams (1947). Three other laboratory strains (Hildenborough, New Jersey SW8, Canet 41) did not reduce fumarate in hydrogen.

Strain El Agheila Z was able to reduce fumarate slowly whether harvested from lactate or malate media. In the latter case *c.* 1 $\mu\text{mole H}_2$ was absorbed/ $\mu\text{mole fumarate}$ reduced ($-\text{Q}_{\text{H}_2}$ *c.* 10), but some CO_2 evolution occurred during the reaction, indicating that the dismutation reaction was proceeding simultaneously. Malate was reduced in place of fumarate, but more slowly ($-\text{Q}_{\text{H}_2}$ *c.* 5).

Formation of lactate

The compounds so far demonstrated in malate metabolism were CO_2 , acetate, fumarate and succinate. Theoretical considerations (see Discussion) required that the dehydration and reduction of malate to succinate should be accompanied by a parallel oxidation of malate to acetate, and intermediates in this step were therefore sought. CTAB-treated cells such as those used to demonstrate fumarase activity (above), showed very slow CO_2 evolution from

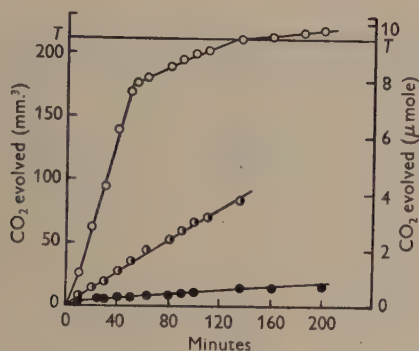


Fig. 1

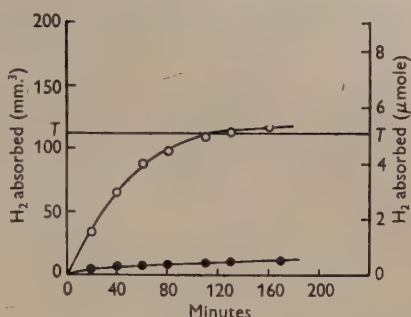


Fig. 2

Fig. 1. CO_2 -evolution curves from *Desulphovibrio desulphuricans* strain El Agheila Z when oxidizing malate or succinate with sulphate. Manometer vessels contained 3.7 mg. dry wt. washed cells (harvested from malate medium) in N_2 at 37° , pH 6.1, CdCl_2 in centre well. $\bigcirc-\bigcirc-\bigcirc$, L-malate ($4.75\mu\text{mole}$). T indicates theoretical volume of CO_2 for molar ratio 1 malate \rightarrow 2 CO_2 . $\bullet-\bullet-\bullet$, succinate ($5\mu\text{mole}$). $\bullet-\bullet-\bullet$, no substrate.

Fig. 2. Reduction of fumarate in hydrogen by *Desulphovibrio desulphuricans* strain California 43:63. Manometer vessels contained 3.1 mg. dry wt. washed cells (harvested from lactate medium) in H_2 at 37° , pH 6.3, NaOH in centre well. $-Q_{\text{H}_2}$ (fumarate) $c. 28$; T indicates theoretical volume of H_2 for fumarate + $\text{H}_2 \rightarrow$ succinate. $\bigcirc-\bigcirc-\bigcirc$ fumarate ($5\mu\text{mole}$), $\bullet-\bullet-\bullet$, no substrate.

malate in the presence of sulphate (Q_{CO_2} $c. 2.6$) which was nevertheless in excess of the blank (Q_{CO_2} $c. 0.9$). The supernatants of such preparations analysed after various time intervals contained significant amounts of lactate; pyruvate was present in traces. Comparisons of the amounts of CO_2 evolved and lactate formed are given in Table 4; there is reasonable agreement with a simple decarboxylation of malate to lactate. Nossal (1951) observed that the malic decarboxylase system of acetone-dried *Lactobacillus arabinosus* required Mn^{++} and cozymase for maximum activity. Neither the rate of CO_2 evolution nor the amount of lactate formed by CTAB-treated El Agheila Z was affected by MnSO_4 ($2 \times 10^{-3}\text{M}$), cozymase (0.8 mg./ml.), both together, cocarboxylase (0.4 mg./ml.) or pyridoxal phosphate (0.2 mg./ml.).

Lactate metabolism

The lactate metabolism of *El Agheila Z* was therefore examined using cells habitually grown in lactate media. Analyses of cultures grown with lactate and excess of sulphate gave final yields of $1\mu\text{mole acetate}/\mu\text{mole lactate}$ utilized (Table 1). Neither growth of bacteria nor CO_2 evolution from suspen-

Table 4. *Formation of lactate from malate by CTAB-treated Desulphovibrio desulphuricans strain El Agheila Z*

Cells were harvested from the malate medium, washed in saline phosphate buffer (pH 6.1) and shaken in N_2 at 37° with $5\mu\text{mole malate}$ and $50\mu\text{g CTAB/mg. dry wt. cells}$ in Warburg manometers. Lactate was determined in the supernatant after incubation. Three experiments recorded.

Malate ($\mu\text{mole}/$ vessel)	Cells (mg./vessel)	Incubation time (min.)	CO_2 evolved (μmole)	Lactate ($\mu\text{mole}/$ vessel)	CO_2 :Lactate (corrected for blank)
5	10	105	1.18	1.18	1:0.74
5	10	125	1.26	1.11	1:0.88
—	10	105	0.36	0.46	—
5	10	90	0.94	1.67	1:1.4
5	10	180	1.07	1.31	1:1.2
—	10	90	0.13	0.26	—
5	5.2	115	1.07	0.54	1:1.3
—	5.2	275	0.49	0.10	—

Mean CO_2 :lactate ratio: 1:1.1 ($\sigma=0.252$).

sions took place with lactate in the absence of sulphate; in the presence of lactate cell suspensions evolved $1\mu\text{mole CO}_2/\mu\text{mole lactate}$ metabolized and $\frac{1}{2}\mu\text{mole sulphide}$ was formed (Table 3). These observations were consistent with the theoretical equation



Lactate oxidation by cell suspensions of *El Agheila Z* showed the widest variation in Q_{CO_2} encountered during this work; values ranging from 50 to 7 were obtained. Low Q_{CO_2} values, when encountered, were maintained over several subcultures of the strain, and were independent of: (a) the age of the cells harvested; (b) addition of any or all of the components of the lactate medium to the test system; (c) conducting the reaction at 30° instead of the usual 37° ; (d) use of N_2 specially freed from O_2 by passage over a heated copper spiral; (e) use of an unwashed cell suspension; (f) manipulation of the suspension in reducing conditions (excess Na_2S); (g) addition of traces of fumarate ($0.5\mu\text{mole}$ (see Discussion)); (h) increase of the iron content of the medium from which the cells were harvested so that the suspension was black with FeS ; (i) decrease of the Na_2S in that medium to $0.5\mu\text{mole/ml.}$; (j) replacement of sulphate by dithionite, this inducing a negative redox potential in the vessel. Hence this variation seems to be a property of the strain examined; the non-halophilic Hildenborough strain behaved more regularly.

Senez (1951) suggested that pyruvate was an intermediate in the formation

of acetate from lactate by *Desulphovibrio desulphuricans*, and this belief was supported (Postgate, 1952*b*) by the fact that the rate of pyruvate oxidation by lactate-grown cells was always greater than or equal to the rate of lactate oxidation. Data illustrating this obtained by using methylene blue reduction in Thunberg tubes as a measure of dehydrogenase activity are given in Table 5. However, unequivocal demonstration of pyruvate as an intermediate was lacking, and attempts were therefore made to demonstrate this by selective inhibition of the hypothetical pyruvate→acetate step. Potassium fluoro-pyruvate (0.25 μ mole/mg. dry wt. cells) did not affect lactate oxidation at all,

Table 5. *Methylene blue reduction by Desulphovibrio desulphuricans strains in presence of certain hydrogen donors*

Washed suspensions of cells harvested from lactate media were incubated *in vacuo* with methylene blue and hydrogen donor in conventional Thunberg tubes. Times for complete decolorization of methylene blue were recorded.

Strain	Hildenborough	El Agheila Z	New Jersey SW 8	New Jersey SW 6
Substrate	Time for complete decolorization (min.)			
Lactate	36	95	62	87
Pyruvate	9	44	44	9
Malate	65	90	∞	121
None	86	116	∞	∞

(∞ signifies > 1000 min.)

and caused no keto-acid accumulation; sodium iodoacetate (3×10^{-3} M) inhibited the blank metabolism and the lactate oxidation almost completely (Q_{CO_2} reduced from 25 to 0.5); 2:4-dinitrophenylhydrazine decreased the rate of lactate oxidation (Q_{CO_2} of 25) to a Q_{CO_2} of 13, but no accumulation of acid phenylhydrazone was observed. Sodium arsenite (0.01 M to 0.0025 M), however, decreased the Q_{CO_2} with lactate by about 50 % and, provided the uninhibited Q_{CO_2} value was greater than *c.* 20, caused significant and reproducible accumulation of pyruvate. No keto-acid was detected in the absence of lactate. Since pyruvate metabolism still took place in the presence of arsenite, quantitative conversion of lactate to pyruvate was not to be expected. However, up to 12 % of the lactate utilized could be accounted for as pyruvate (Table 6). Since the demonstration of pyruvate formation from lactate was of some general importance, it was desirable to repeat these experiments with a second strain of these bacteria. Table 6 includes some experiments showing pyruvate accumulation by arsenite-inhibited cells of the Hildenborough strain harvested from a lactate medium.

Pyruvate formation from malate was not successfully demonstrated because arsenite had a stronger inhibitory effect on the initial attack on malate than on pyruvate oxidation: even at 0.0001 M it decreased the Q_{CO_2} by *c.* 50 %; no keto-acid accumulated.

Pyruvate oxidation

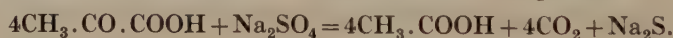
The metabolism of pyruvate was already known to be complex owing to the apparent existence of two pathways of 'sulphate-free' metabolism as well as the oxidation in the presence of sulphate. It is hoped to report a detailed study of these reactions at a later date; in the present work the study of pyruvate metabolism was restricted to establishing the stoichiometry of its reaction with sulphate. Cultures of strain El Agheila Z grew to higher cell densities with

Table 6. *Formation of pyruvate from lactate by arsenite-inhibited Desulphovibrio desulphuricans strains*

Cell suspensions were shaken in Warburg manometers (37°, N₂) with Na lactate (5 μ mole) Na₂SO₄ (20 μ mole) Na arsenite, 0.01 M, pH 6.9, for various times and the supernatant analysed for pyruvate and residual lactate.

Strain	Cell density (mg./vessel)	Incubation time (min.)	Pyruvate formed (μ mole)	Lactate used (μ mole)	Pyruvate as % (w/w) lactate
El Agheila Z	4.7	290	0.066	—	—
	3.9	155	0.055	0.60	9.5
	3.4	30	0.055	0.45	12
Hildenborough	2.75	40	0.11	0.95	11.6
	3.2	40	0.69	2.12	32.5

pyruvate + sulphate than with equimolar lactate or malate (see Senez, 1951), and analysis of cultures grown in these conditions gave a final yield of 1 μ mole acetate/ μ mole of pyruvate utilized (Table 1). Cells harvested from lactate medium oxidized pyruvate without a lag with a Q_{CO_2} of c. 45 (range: 80 to 40); the quotient was never less than that with lactate and often exceeded it; 1 μ mole CO₂ was evolved/ μ mole pyruvate oxidized and $\frac{1}{4}$ μ mole S²⁻ was formed (Table 3). These observations are consistent with the equation:

*Effect of carbon compounds on sulphate reduction in hydrogen*

Senez (1954*b*) reported that the rate at which heterotrophically-grown cells of *Desulphovibrio desulphuricans* strain Canet 41 reduced sulphate in hydrogen was enhanced by pyruvate or lactate, in spite of the fact that the carbon compounds were oxidized simultaneously. Similar effects were demonstrated with malate-grown El Agheila Z, provided the $-Q_{H_2}^{SO_4}$ was determined with cells that had been pre-incubated for some time in N₂ with excess Na₂SO₄ to bring stored carbon compounds to a low concentration. Marked stimulation of sulphate reduction occurred regularly if small amounts of fumarate or malate were present, but succinate, lactate and pyruvate did not usually have an effect (Table 7).

Fisher, Krasna & Rittenberg (1954) showed that a lag in the hydrogenase activity of *Proteus vulgaris* was reduced by metabolic substrates such as glucose or fumarate, as well as by dithionite, and attributed this to removal

Table 7. Influence of certain carbon compounds on the rate of substrate reduction with hydrogen by *Desulphovibrio desulphuricans* strain El Agheila Z

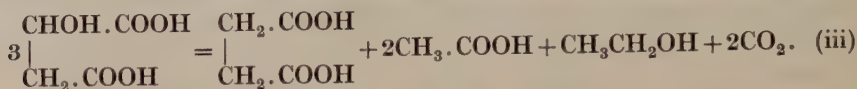
Experiment ... Supplement	1	2	3	4
—	16	17	12	26
Malate	24	33	17	35
Fumarate	26	46	20	30
Succinate	17	17	11	—
Lactate	—	22	12	27
Pyruvate	—	17	10	42

Experiment	1	2
Substrates				
Sulphate			46	43
Sulphate and fumarate			77	72
Dithionite			154	111
Dithionite and fumarate			155	109

A scheme for malate metabolism in *Desulphovibrio desulphuricans*

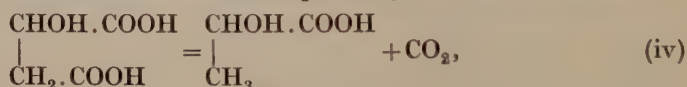
$$2 \begin{array}{c} \text{CHOH} \cdot \text{COOH} \\ | \\ \text{CH}_2 \cdot \text{COOH} \end{array} \rightleftharpoons 2 \begin{array}{c} \text{CH} \cdot \text{COOH} \\ || \\ \text{CH} \cdot \text{COOH} \end{array} + 2\text{H}_2\text{O}, \quad (\text{i})$$
$$2 \frac{\text{CH}_3.\text{COOH}}{\text{CH}_3.\text{COOH}} + \frac{\text{CHOH}.\text{COOH}}{\text{CH}_2.\text{COOH}} + \text{H}_2\text{O} = 2 \frac{\text{CH}_2.\text{COOH}}{\text{CH}_2.\text{COOH}} + \text{CH}_3.\text{COOH} + 2\text{CO}_2. \quad (\text{ii})$$

Acetate, succinate and CO_2 could in theory be formed by a second type of breakdown in which ethanol would be expected as an additional end product:

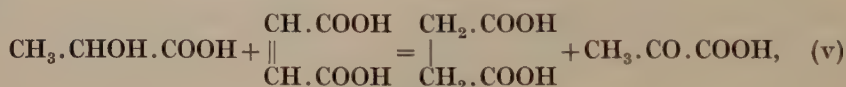


This reaction requires, however, not only that ethanol be formed but that 3 malate yield 2 acetate + 1 succinate + 1 ethanol + 2CO_2 . This bears no relation to the analytical figures obtained, though traces of material that may have been ethanol were in fact found in the products of malate breakdown without sulphate. Thus, if this reaction occurs at all, it is unimportant, and equations (i) and (ii) probably give the most satisfactory account of malate dismutation.

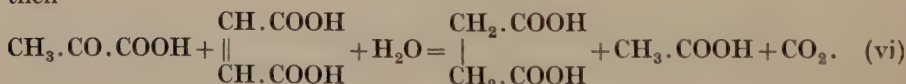
The reduction of the two fumarate molecules to succinate is coupled, in the absence of sulphate, with oxidation of malate to acetate, and the formation of lactate by CTAB-treated cells indicates that lactate is involved in this step. The formation of pyruvate from lactate by arsenite-inhibited cells provides direct evidence supporting the view that pyruvate is an intermediate in lactate breakdown, and, though pyruvate formation from malate was not observed, it is reasonable to suppose that pyruvate formation in fact took place since lactate formation was demonstrated. Thus the oxidation of malate which parallels reduction of fumarate to succinate probably follows the courses:



then

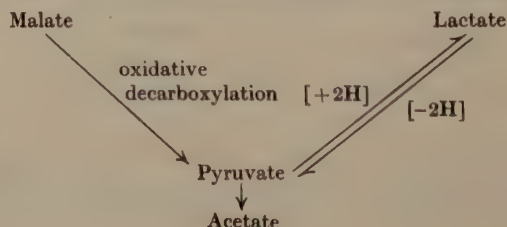


then



Oxido-reductions of the kind represented in (v) and (vi) are well known in other bacteria (see Stephenson, 1949). Equations (i), (ii), (iv), (v) and (vi) can be combined to form the scheme of malate metabolism shown in Fig. 3.

Lactate and pyruvate have been written as successive stages in the oxidative side of the scheme shown in Fig. 3. There is, however, no evidence from this work against the possibility that pyruvate is the initial product and that lactate is in equilibrium with it; this is thought to occur in the malic decarboxylase system of *Lactobacillus arabinosus* (Korkes, Campillo & Ochoa, 1950).



So far the discussion has been restricted to malate breakdown without sulphate, but there is strong evidence that similar reactions take place when sulphate is present. The evidence may be summarized: (i) succinate accumulates transiently during oxidation of malate in the Warburg manometer; (ii) cultures of bacteria which have not oxidized all the available malate contain an excess of acetate above the theoretical value for straightforward oxidation by sulphate; hence acetate formation uncoupled with sulphate reduction is occurring; (iii) succinate, fumarate and lactate were demonstrated chromatographically in such cultures—though no data concerning amounts present were available.

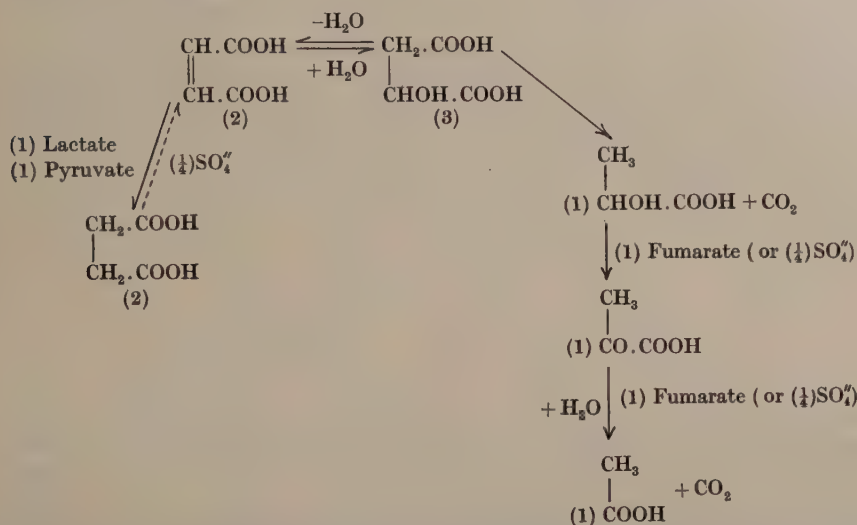


Fig. 3. Scheme for malate metabolism in *Desulphovibrio desulphuricans*. The numbers preceding the names or structures of compounds indicate the number of molecules participating in each metabolic step.

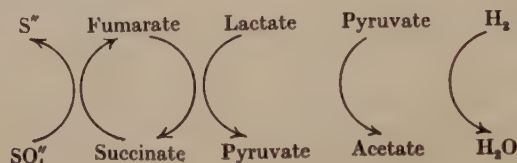
This view leads to the rather surprising conclusion that there is only one reaction in the metabolism of malate for which sulphate is essential, that is, the oxidation of succinate to fumarate. Obviously, sulphate reduction can be linked to lactate oxidation and pyruvate oxidation; indeed, it is essential for the former if malate or fumarate are absent, but fumarate can act as an alternative hydrogen acceptor to sulphate for all reactions except its formation from succinate, and in fact does so even when sulphate is present.

The role of fumarate as a hydrogen acceptor

Sisler & ZoBell (1951) postulated a reductive fission of fumarate to yield acetate; the present work indicates that the reduction proceeds only as far as succinate. The reaction is not necessarily associated with malate metabolism since (a) Sisler & ZoBell first observed it in autotrophically grown cells, (b) in the present work it was observed in lactate-grown cells of Sisler & ZoBell's strain, (c) both lactate- and malate-grown cells of *El Agheila Z* conducted the reaction. It seems probable, then, that fumarate

reduction in hydrogen is a property common to all hydrogenase-containing strains of *Desulphovibrio desulphuricans* and that the failure to demonstrate it with strains Hildenborough, New Jersey SW8 and Canet 41 was due to the reaction proceeding at a rate lower than that of the 'blank' hydrogen absorption; the strain California 43:63 may be exceptional only in that it conducts the reaction rapidly.

The argument outlined in the preceding section establishes that fumarate can also act as hydrogen acceptor for the oxidation of lactate and of pyruvate. The only reaction in the sequence (Fig. 3) proposed for which sulphate is essential is the oxidation of succinate. If succinate oxidation proceeds by way of fumarate, as the sequence above assumed, one can envisage a scheme in which the oxidation of succinate would be linked to sulphate reduction, and the reduction of fumarate would be linked with the oxidation of hydrogen, lactate or pyruvate. A fumarate-succinate cycle would thus be part of the electron-transfer system between the oxidation of hydrogen donors and the reduction of sulphate:



Such a view would be consistent with the report of Senez (1954*a*) of the presence of malic, fumaric and succinic dehydrogenase systems in lactate-grown El Agheila Z. This hypothesis is consistent with the facts reported here, but before it can be proved or disproved it requires a more detailed examination of the precise factors which link carbon oxidation to sulphate reduction. It is, however, pertinent to point out that the analogous system in all other organisms, the succinoxidase system, is cytochrome linked, and Postgate (1954*a, b*) showed that the cytochrome of *Desulphovibrio desulphuricans* was directly involved in sulphate reduction.

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Patterns of Enzymic Adaptation in Species of the Genus *Azotobacter*

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SUMMARY: Fourteen strains of the *Azotobacter agile*-*A. vinelandii* group were classified into one or the other of the above species by morphological, cultural and growth characteristics. The groups were then surveyed by a study of oxidative reactions of whole cell suspensions on some intermediates of the tricarboxylic acid (TCA) cycle: acetate, malate, succinate, α -ketoglutarate and citrate.

All strains grew readily in a nitrogen-free medium. Organisms that were morphologically large rods or ovoid cells produced a green water-soluble pigment in an iron-deficient medium, and did not utilize mannitol or nitrate in the absence of another carbon or nitrogen source. Strains composed of smaller rods also produced the green pigment, and would utilize mannitol and nitrate. The first group was classified as *Azotobacter agile*, and the second as *A. vinelandii*.

Q_{O_2} (N) values on the TCA substrates tested did not indicate group variation. However, 15-24 hr. cells of the *Azotobacter agile* group as a unit did oxidize succinate with a shorter lag period (not greater than 30 min.) than strains of the *A. vinelandii* group (not less than 50 min.). A similar response to malate was noted, but in contrast most strains of *A. vinelandii* oxidized α -ketoglutarate after a shorter lag than did strains of *A. agile*. Citrate oxidation was variable among strains; all organisms but *A. vinelandii* O were apparently inhibited by the chelation of magnesium (and possibly calcium) by citrate. The physiological and oxidative responses of freshly isolated strains did not differ markedly from those of laboratory cultures of the same species.

For many years now the role of physiological methods in establishing the taxonomic position of a bacterial strain has been recognized and accepted, but some investigators are often troubled by the exclusive use of what appears to them to be rather crude biochemical tests in determining an organism's 'physiology'. Undoubtedly at one time or another, most bacterial physiologists have wondered whether more modern techniques of enzyme specification could not be applied to taxonomic questions. The difficulties of doing so on the large scale demanded by the survey of hundreds of bacterial isolates are recognized to be formidable though certainly not insurmountable. For the time being, at least, the conventional methods represented by such a physiological description as 'acid and gas on lactose', for identification and classification, must remain the basic set for initial screening. But perhaps it is not too early to begin studies in which the more advanced quantitative methods are applied to special problems that have been defined through the conventional techniques.

As an example of such an approach we have recently examined some of the enzymic properties of representative strains of the *Azotobacter agile-vinelandii* group. ('Group' is used in this paper as a convenient term to include strains

labelled either *A. agile* or *A. vinelandii* without any implication as to whether the group comprises one or more species; see Discussion.) For two reasons this group appears to offer a reasonably significant test case. First, the results of traditional methods have led to disagreement as to whether one or two species should be recognized; secondly, a considerable amount of information concerned with the enzymic properties of a few strains exists. Extension of such studies to other strains might help to decide whether the few gross morphological and physiological differences observed between members of the two groups can be correlated with more subtle differences in specific enzyme properties.

METHODS

Organisms used. Fourteen strains were used in this study, nine of which had been classified previously as *Azotobacter agile* or *A. vinelandii*. A tenth strain was received unclassified from Kansas State College and identified as *A. vinelandii* by morphology and by the following growth characteristics: ability to grow readily in a nitrogen-free liquid medium with the formation of a green water-soluble pigment when this medium is deficient in iron. The remaining four strains were fresh isolates of *A. agile* as determined by cell size and shape and by the foregoing growth properties. Source and classification of all strains are listed in Table 1.

The organisms were maintained on Burk's mineral salts medium with 2% (w/v) sucrose (Wilson & Knight, 1952). A 2-3% (v/v) inoculum was introduced into 100 ml. medium in a 500 ml. Erlenmeyer flask and shaken 350 r.p.m. at 30° for 15-24 hr. Colonial characteristics were observed from growth on the same medium solidified with 1.5% agar.

Table 1. *Source of strains of azotobacter*

<i>Azotobacter agile</i>		<i>Azotobacter vinelandii</i>	
Strain	Source	Strain	Source
9040	American Type Culture Collection; R. L. Starkey, donor	7492	American Type Culture Collection; D. Burk, donor
7494	ATCC; N. R. Smith, donor	7496	ATCC; N. R. Smith, donor
4-4	Stock culture of the University of Wisconsin; originally from Dr H. A. Barker, University of California	7484	ATCC; N. R. Smith, donor
S-1	Isolated from Lake Mendota, Madison, Wisconsin by J. Schutter	O	Original strain from the University of Wisconsin
S-2	Lake Mendota, Madison, Wisconsin	3	Stock culture of the University of Wisconsin; originally from R. L. Starkey
S-3	Lake Mendota, Madison, Wisconsin	K	From T. G. G. Wilson; originally from Rothamsted Experimental Station, England
S-4	Lake Mendota, Madison, Wisconsin	K-S4	From J. O. Harris, Kansas State College

Nitrate utilization. Because Green & Wilson (1953) observed that *Azotobacter vinelandii* utilizes potassium nitrate in the absence of molecular nitrogen, while *A. agile* 4.4 does not, all strains were tested. A modification of the method of Horner & Allison (1943) was used; 125 ml. flasks each containing 15 ml. Burk's media with 2% (w/v) sucrose and 300 $\mu\text{g./ml.}$ nitrate as KNO_3 were inoculated with 0.1 ml. active culture from a nitrogen-free medium. The flasks in 200 cm. vacuum desiccators were maintained under 0.2 atm. of oxygen and 0.8 atm. of hydrogen, the latter to prevent any fixation of atmospheric nitrogen. The desiccators were shaken 100 strokes/min. on a reciprocal shaker at 30°. After 4 days the pH value was determined. Evidence of visible growth was accompanied by rise in pH value owing to disappearance of nitrate ion. This simple test was occasionally checked by actual measurement of the amount of nitrate that disappeared by the method of Nelson, Kurtz & Bray (1954).

Manometric experiments. Organisms to be tested for oxidative activity were removed at 15–24 hr. from liquid cultures by centrifugation, washed once by suspending in 10 times the cell volume of cold 0.2% (w/v) KCl solution, recentrifuged and resuspended in a fresh portion of KCl. Total nitrogen was determined by a semimicro-Kjeldahl method.

Standard manometric procedures were used to measure rate of oxygen uptake on sucrose and the tricarboxylic acid (TCA) intermediates: acetate, malate, succinate, α -ketoglutarate, and citrate. $Q_{O_2}(\text{N})$ values ($\mu\text{l.O}_2/\text{hr./mg. N}$), corrected for the low endogenous respiration, were calculated after the end of the lag period from the rate of oxygen uptake on the linear part of the curve. Unless otherwise stated, the contents of Warburg vessels were: a volume of cell suspension containing 60–100 $\mu\text{g. N}$; 80 μmole potassium phosphate buffer at pH 7.0; 2 μmole MgSO_4 ; water to make a total volume of 3.2 ml.; 40 μmole substrate in the side arm; 0.1 ml. 20% KOH in the centre well. The atmosphere was 50% O_2 plus 50% He, and the temperature was 36°. For general characteristics of oxidations by the azotobacter under these conditions see Williams & Wilson (1954).

RESULTS

General morphological, cultural and physiological characteristics

A primary separation of *Azotobacter agile* strains from those of *A. vinelandii* could be made on the basis of the morphology of young cells. The *A. agile* organisms were usually ovoid or spherical, measuring 2.0×2.0 – 4.5μ . The *A. vinelandii* organisms were smaller rods not exceeding 1.5μ . in width and 3.0μ . in length (see Jensen, 1954). The colonies of the strains of *A. vinelandii* were gummy in contrast with the drier, smaller colonies of *A. agile*. After an incubation period of 1 week no strains of *A. agile* had grown visibly in liquid Burk's medium in which 2% mannitol had been substituted for sucrose; all the *A. vinelandii* strains grew within 24 hr. Likewise all strains of *A. vinelandii*, but none of *A. agile*, grew when KNO_3 was the sole source of nitrogen.

Pigment formation varies according to the mineral content of the medium

particularly that of iron and molybdenum (Wilson & Knight, 1952). For test we have used Burk's medium without Fe+Mo solution. Under these circumstances the *Azotobacter vinelandii* strains turned the medium a bright green, whereas the colour formed by *A. agile* was less intense. On ageing in the refrigerator the agar slopes of *A. agile* became reddish to purple, whereas those of *A. vinelandii*, which were originally greenish yellow, faded.

Oxidative properties and enzymatic adaptation

Table 2 summarizes the rates of respiration of the fourteen strains on typical TCA intermediates. Figs. 1 and 2 present the adaptative patterns for typical representatives of each type (similar figures for *Azotobacter vinelandii* O and for *A. agile* 4.4 are included in previous publications (e.g. Repaske & Wilson, 1953; Williams & Wilson, 1954). No consistent differences among the rates

Table 2. *Rates of respiration by azotobacter strains with various substrates*

Strain	Substrate					
	Sucrose	Acetate	Malate	Succinate	α -keto-glutarate	Citrate*
	$Q_{O_2}(N): \mu l. O_2/hr./mg.N$					
	<i>Azotobacter vinelandii</i>					
O	7,900	13,600	12,300	11,600	11,800	5,000
K	8,300	16,800	16,200	20,200	14,900	3,300
3	3,800	15,600	13,500	17,600	21,000	1,500
7492	5,700	11,500	8,100	15,500	11,200	0
7484	6,000	8,200	9,400	8,100	8,700	Not tested
7496	8,700	10,700	14,600	14,300	11,200	0
K-S4	7,000	9,300	8,900	10,900	11,700	0
	<i>Azotobacter agile</i>					
4.4	10,700	13,500	16,500	17,600	12,600	3,600
9040	13,100	22,200	17,500	18,400	11,300	1,900
7494	12,300	13,900	13,900	13,200	10,400	2,800
S-1	7,800	12,400	9,600	13,600	9,500	2,200
S-2	13,400	14,500	20,700	17,900	6,600	1,100
S-3	13,200	16,800	15,300	13,600	4,300	2,600
S-4	14,800	16,500	17,100	16,700	3,500	Not tested

* Values obtained under special conditions cited in text.

of respiration could be correlated with the type of organism, but a study of results similar to those shown in Figs. 1 and 2 for the entire 14 strains suggested certain differences in the pattern of adaptation to a few of the substrates.

Over a period of 2 years experiments have been made to determine whether such patterns were consistent enough to allow any helpful differentiation between the two types. From the results we have concluded that the patterns of adaptation, at least with these 14 strains, are reasonably stable so that behaviour on succinate and to a lesser extent on α -ketoglutarate and malate can differentiate between *Azotobacter agile* and *A. vinelandii*. Fig. 3 illustrates typical findings with 6 strains; the curves for the others showed similar clustering about the proper group of lines in this figure. Analogous though

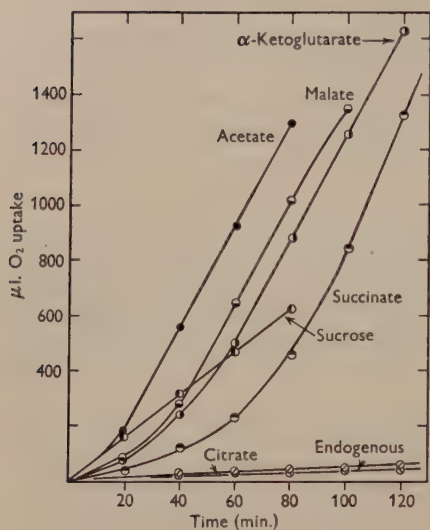


Fig. 1

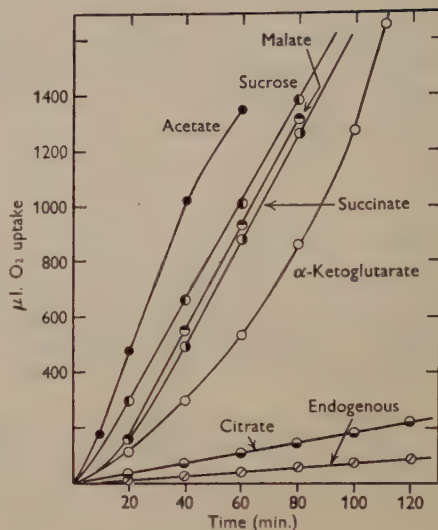


Fig. 2

Fig. 1. Respiratory activity of sucrose-grown *Azotobacter vinelandii* 7492 on sucrose and five substrates of the tricarboxylic acid cycle.

Fig. 2. Respiratory activity of sucrose-grown cells of *Azotobacter agile* S-1 on sucrose and five substrates of the tricarboxylic acid cycle.

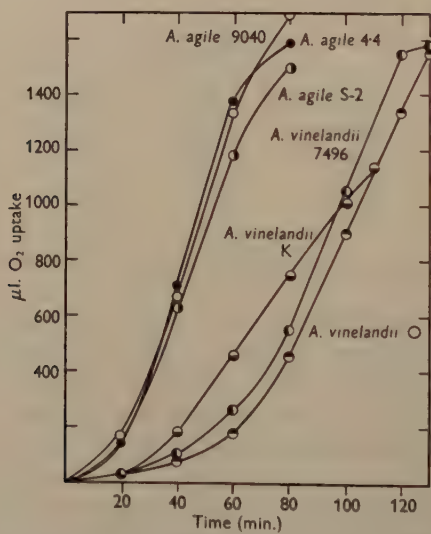


Fig. 3. Respiratory activity of sucrose-grown cells of *Azotobacter vinelandii* O, K, and 7496 and *Azotobacter agile* 9040, 4-4, and S-2 on sodium succinate.

not so clear-cut differences were obtained with α -ketoglutarate and malate; with the strains we have used, however, succinate certainly would be the substrate of choice for screening.

The results with citrate call for special mention, particularly, since observations made in this laboratory with citrate first suggested this study. Extended experiments with the 14 strains, however, did not lend support to our original view that it might be used as a differentiating substrate. While these experiments were in progress, we learned through private communication with Dr N. Levy of Imperial Chemical Industries Ltd. that an important factor determining whether citrate (and probably other TCA intermediates) is utilized by *Azotobacter* spp., and probably other species, is the ratio of citrate:Mg⁺⁺. Dr Levy kindly furnished us with a summary of his results* prior to their publication so that we were able to test the influence of this factor on 12 of the 14 strains. Three distinct responses, which were independent of the type of organism, were observed when the ratio citrate:Mg⁺⁺ was varied: (a) one strain (*A. vinelandii* O) adapted to citrate independently of the citrate:Mg⁺⁺ ratio; (b) three strains (*A. vinelandii* 7492, 7496, K-S4) did not adapt during these experiments (210 min.) even with the lowest citrate:Mg⁺⁺ ratio; (c) the remaining strains did or did not adapt, depending on the citrate: Mg⁺⁺ ratio (see Tables 2 and 3).

Because a lowering of the citrate:Mg⁺⁺ ratio permitted citrate oxidation by 10 of the 14 strains, it was reasoned that an inhibitory factor in previous experiments had been the chelation of ions such as Mg⁺⁺ and Ca⁺⁺ by an excess citrate, rendering these unavailable for TCA cycle oxidations. Harris (1954) suggested the same type of inhibition of citrate oxidation by *Azotobacter chroococcum*; since calcium is required by the organism for maximum respira-

* We thank Dr Levy and his associates not only for supplying us with this information in time for testing on the collection we used but also for the courtesy of allowing us to quote from a letter:

'For *A. vinelandii* (Rothamsted strain 5921) response to citrate is governed by (i) citrate: magnesium ratio in the medium and (ii) concentration of Mg⁺⁺ and Ca⁺⁺ in the cells, in close conformity with a membrane equilibrium model for Mg(Ca) transfer, based on the 1:1 citrate: kation complex and citric acid dissociation. In growth experiments using 1 per cent mannitol medium with 5-40 p.p.m. Mg and c. 1 p.p.m. soluble Ca, citrate stimulates fixation when the citrate:Mg ratio is not much above 1, inhibits completely above 5 and extends the lag (with ultimate stimulation) at intermediate ratios. Raising soluble Ca to 10 p.p.m. roughly doubles these thresholds, i.e. in given cases adequate Ca will convert inhibition to normal or stimulated growth. For a range of organic anions (tartrate, malate, citrate, acetyl acetate, E.D.T.A.) inhibitory power rises with the stability constant of the 1:1 complex.

Oxidation of citrate by resting cells can also be varied from complete to slight according to (i) and (ii) above. The oxygen-uptake curves for acetate, malate, succinate, and citrate under equivalent conditions show "lags" rising in that order from zero to 4-5 hr. This "lag" could be explained, not by enzyme adaptation or anion permeability change, but by initial rapid membrane equilibrium (extraction or other immobilization of Mg⁺⁺ and Ca⁺⁺) followed by re-mobilization and accelerating oxidation as the anion is consumed. Acetate has virtually no chelating power and is attacked without delay; citrate has the highest stability constant in this series and the longest "lags". It is thus possible to set up with a single strain parallels to the behaviour of the different groups you mention.'

Obviously, concentrations and/or ratios of Mg⁺⁺ and citrate other than those used by ourselves (Table 3) might lead to variations in the results, for example, the recalcitrant strains of *A. vinelandii* might be induced to use citrate.

Table 3. *Influence of citrate:Mg⁺⁺ ratio on respiratory activity of whole azotobacter cells*

Strain	Citrate:Mg ⁺⁺ ratio	Citrate (μ mole)	Mg ⁺⁺ (μ mole)	Lag period (min.)	Q _{O₂} (N) (μ l. O ₂ /hr./mg.N)
<i>Azotobacter vinelandii</i>					
O	20:1	40	2	10	5000
	10:1	20	2	10	5900
	5:1	10	2	10	4500
	2:1	8	4	10	4500
	1:1	6	6	10	3800
	5:0	5	0	10	3100
K	20:1	40	2	180-210	—*
	2:1	8	4	180-210	—
	1:1	10	10	160	3200
	1:1	14	14	170	865
	1:2	10	20	170	1800
	1:1	6	6	180-210	—
3	20:1	40	2	210	—
	1:1	10	10	210	—
	1:1	6	6	200	1470
<i>Azotobacter agile</i>					
9040	20:1	40	2	180-210	—
	10:1	20	2	180-210	—
	5:1	10	2	180-210	—
	3:1	60	20	180-210	—
	2:1	8	4	150	1800
	1:1	6	6	150	2000
	1:1	40	40	180-210	—
	5:0	5	0	180-210	—
4-4	20:1	40	2	180-210	—
	1:1	10	10	150	3400
	1:1	18	18	170	3800
7494	20:1	40	2	210	—
	1:1	10	10	160	2800
S-1	20:1	40	2	210	—
	1:1	10	10	210	—
	1:1	6	6	190	2180
S-2	20:1	40	2	210	—
	1:1	10	10	160	850
	1:1	6	6	200	1100
S-3	20:1	40	2	210	—
	1:1	6	6	200	2550

* Oxygen uptake less than 50 μ l./hr.

tory activity, the lowering of the rate of oxygen uptake may arise from the formation of 'insoluble calcium citrate salts'. If a magnesium (calcium) deficiency were a factor in the low rate of citrate oxidation, it was reasoned that the presence of excess citrate would lower respiratory rate on other substrates oxidized through the TCA cycle. This was substantiated in part by the addition of acetate to Warburg vessels containing cells that had been preincubated with 40 μ mole of citrate and 2 μ mole of MgSO₄ for approximately 1 hr. (Table 4).

DISCUSSION

Before summarizing conclusions to be drawn from this study, two side observations of the research should be mentioned:

(1) Adaptive patterns of the strains have been tested over a 2-year period and found to be constant. Even more important, freshly isolated strains of the agile group possessed the same adaptive patterns as did stock strains which had been kept on laboratory media for many years. These data suggest that the patterns found are stable and representative of the organism in nature and not a laboratory artefact.

Table 4. *Influence of preincubation with citrate on the respiratory activity of azotobacter cells on acetate*

Strain	$Q_{O_2}(N)$ values on acetate	
	Preincubation without citrate	Preincubation with 40 μ mole citrate
	$(\mu l. O_2/hr./mg. N)$	
	<i>Azotobacter agile</i>	
4-4	18,900	7,400
S-1	12,400	5,400
S-2	17,600	11,700
7494	11,200	6,900
<i>Azotobacter vinelandii</i>		
O	14,600	4,500
K	16,500	5,800
3	13,200	7,100
K-S4	16,500	4,400

(2) The difficulty of comparing lag times without rigid control of the age of the organisms under observation can be ascertained from the data of Williams & Wilson (1954), in which lag times for *Azotobacter vinelandii* O on succinate and α -ketoglutarate were shown to vary widely with the age of the organisms from 6 to 60 hr. However, since the present study was in part to evolve a simple technique that would facilitate identification of a questionable strain of *Azotobacter* an age variation of 15–24 hr. was allowed in the experiments. Within this range only succinate emerged as a clear 'indicator' substrate: *A. agile* organisms 15–24 hr. old had a lag period of 30 min. or less, whereas *A. vinelandii* strains 15–24 hr. old had lag periods of at least 50 min. Also within this range, a trend of lag differences between species on oxidation of malate and α -ketoglutarate was noted; but a few strains of one species have lag times overlapping those of strains of the other species when comparisons are made between organisms that may vary as much as the allowed 10 hr. in age.

Independent of the particular mechanism concerned with adaptation, be it physical or biological, certain major conclusions appear permissible from these studies. First, ability to adapt to succinate and to a lesser extent to malate and α -ketoglutarate might be useful criteria for differentiating between

members of these two groups of *Azotobacter* spp. Secondly, the observations encourage the belief that similar and additional studies of this type (e.g. examination of cell-free extracts for characteristics, including concentration of specific enzymes) might be a powerful tool for more precise definition of an organism's physiology. Such definition should be particularly useful in classification of doubtful cases found in taxonomic studies of this and other micro-organisms. Whether the observed differences are sufficient for division into species in the present example is not a decision for the physiologist but for the expert in taxonomy. The physiologist can only ask that, if his field is to be used in making such decisions, recognition be taken of the advances in this area. Thus more modern methods will be increasingly utilized, methods that he believes might be more powerful and selective than those at present represented in *Bergey's Manual of Determinative Bacteriology* and other taxonomic manuals.

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The Nutritional Requirements of Isolates of *Labyrinthula* spp.

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SUMMARY: The *Labyrinthula* isolates of Watson will grow over a wide range of pH values in a medium of which the inorganic constituents reflect their marine origin. All are obligately marine, though the less exacting requirements of *L. macrocystis* var. *atlantica* suggest an estuarine habitat. Although only *L. minuta* var. *atlantica* requires an amino acid (L-leucine) as a growth factor, no isolate grew well on substrates other than amino acids. All require thiamine. Organic requirements may be obtained from host plants.

Labyrinthula Cienkowski (1863) is a genus of uncertain affinities, usually considered among the Myxomycetes. It is best known as including the probable causative organism of the wasting disease of eel grass, *Zostera marina* (Renn, 1935). Pure cultures of some members of the genus *Labyrinthula* have only recently been isolated by Dr S. W. Watson (to be published). The availability of pure cultures has made possible study of nutritional requirements. These studies were motivated not only by the specific economic importance of *Labyrinthula* spp. but by the interest which attaches to the nutrition of parasites and of marine micro-organisms in general. The latter because of the paucity of precise data on the requirements of an ecologically important group; the former because parasites may require growth factors available only in the intimate host-parasite relationship. Such parasites are uniquely useful biochemical tools.

Dr S. W. Watson has isolated three strains of *Labyrinthula*: *L. vitellina* var. *pacifica* (P), *L. macrocystis* var. *atlantica* (A), and *L. minuta* var. *atlantica* (M). *L. vitellina* var. *pacifica* has a steroid growth factor requirement specifically unlike the steroid requirements of the few micro-organisms which share this characteristic (pleuropneumonia-like organisms, *Trichomonas* spp., *Paramoecium aurelia*, *Saccharomyces cerevisiae*; for references see Vishniac & Watson, 1953; Vishniac, 1955). This nutritional requirement for a steroid by *L. vitellina* var. *pacifica*, together with preliminary observations on the maintenance of all three isolates, was reported by Vishniac & Watson (1953). The present paper reports the results of experiments on inorganic and organic nutritional requirements of the three *Labyrinthula* isolates above mentioned.

METHODS

Cultures were maintained in screw-capped 'Kimble' culture tubes containing 5 ml. of the medium given in Table 1. The ingredients of this medium were completely dissolved in distilled water to one-half of the final volume, and

the pH value adjusted to 7·8–8·0 with potassium hydroxide, before the addition of agar and thiamine and dilution to final volume. Isolate P required in addition 0·5 mg. cholesterol/100 ml.; this cholesterol was added as an ethanolic solution to the still-boiling medium after solution of the agar. Gelatin hydrolysate was prepared as previously described (Vishniac & Watson, 1953). The stock solution of iron used contained $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 24·9 mg./ml. (Fe^{++} , 5 mg./ml.); EDTA, 30 mg./ml.; $(\text{NH}_4)_2\text{CO}_3$, 20 mg./ml.

Table 1. *Labyrinthula medium*

100 ml. contains:

NaCl	2·5 g.	Zn (as $\text{ZnSO}_4 \cdot 6\text{H}_2\text{O}$)	2·0 mg.
KCl	0·1 g.	Mn (as $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$)	2·0 mg.
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0·5 g.	Fe^\dagger	0·2 mg.
KH_2PO_4	0·01 g.	Co (as sulphate)	0·02 mg.
CaCO_3	0·02 g.	Cu (as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$)	0·002 mg.
EDTA*	0·05 g.	B (as boric acid)	0·02 mg.
$(\text{NH}_4)_2\text{CO}_3$	0·02 g.	Mo (as sodium molybdate)	0·02 mg.
†Gelatin hydrolysate	0·2 g.		
Agar	0·1 g.	Thiamine HCl	20 μg .

* EDTA = Ethylenediamine tetracetic acid = 'Versene', free acid; Bersworth Chemical Co., Framingham, Mass. U.S.A.

† Prepared as by Vishniac & Watson (1953).

‡ As stock solution containing: $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 24·9 mg./ml. (Fe, 5·0 mg./ml.); EDTA, 30 mg./ml.; $(\text{NH}_4)_2\text{CO}_3$, 20 mg./ml.

Experimental media were based on the formula given in Table 1, with the omission of the constituent being studied. Some of the experiments on inorganic nutrition were, however, conducted with a basal medium more nearly resembling that of Vishniac & Watson (1953). Reagent grade chemicals were used. Experimental media were dispensed in 10 ml. lots in 25 ml. glass-capped Erlenmeyer flasks. After sterilization for 12 min. at 120° and inoculation, the flasks were sealed between Pyrex kitchen trays with transparent cellulose tape and incubated for 4–9 days at 20–21°. Growth was followed by visual estimation until the end of the incubation period, when the optical densities of the cultures were read by a Klett-Summerson photoelectric colorimeter with no. 42 filter.

The media used contained 0·1 % (w/v) Difco Bacto agar, an ingredient which contributes unknown chemical contaminants. The use of agar could be obviated by stirring the cultures mechanically on a shaking or rotating apparatus or by vigorous aeration. Such stirring is difficult to provide while simultaneously maintaining sterility, an all-glass culture assembly, and the temperature control considered necessary. The adequacy of the medium developed, in the absence of agar, was tested by growing each of the isolates with stirring by one or more of the methods mentioned.

All isolates grow equally well when incubated at 20° or 25° in tubes of stock medium for 5 days. Isolate M grows as well at 30°, while isolate A grows only slightly or not at all and isolate P not at all at this temperature. Dr S. W. Watson (personal communication) found that isolate A on serum sea water

agar grew as well at 30° as isolate M. All isolates are killed by exposure to 36° for 5 days. Since room temperature in our laboratory frequently exceeds 30° during the summer and during the period when the heating system is in use, it seemed advisable to use a refrigerated incubator for these studies. The temperature 20–21° was chosen because an incubator which could be controlled at this temperature was continuously available.

Preliminary experiments (and unpublished data of Dr Watson) indicated that the pH values of the medium could not be lowered below 7·8 without decreasing or abolishing growth. For this reason nearly all experiments were conducted at pH 7·8–8·0. The pH value of media was routinely adjusted, using bromthymol blue and metacresol purple indicators. After the development of the medium given above, the pH response of all isolates was again investigated, using tubes of stock medium adjusted to pH 7·8–8·0 as usual and subsequently adjusted with sulphuric acid or potassium hydroxide (by using a Beckman model G pH meter) over the range pH 3·05–8·52 in steps of approximately 0·5 pH unit. In this experiment growth was limited by the appearance of a slight (pH 8·00) or heavy (pH 8·2) precipitate in the medium at the alkaline end of the range and abolished by changes in the agar on autoclaving at the acid end (pH 4·5 and below). Slight, possibly not significant, maxima were observed: isolate A, pH 7·50; isolate M, pH 6·60; isolate P, pH 7·50. Growth tends to bring the medium to a reaction slightly above neutrality.

RESULTS

Inorganic requirements

All three isolates are obligately marine. The requirement for sodium chloride is illustrated in Fig. 1. No growth was made by isolates P and M when potassium chloride was supplied at 5·0 mg./100 ml. or less. Potassium chloride at 50 mg./100 ml. gave excellent growth, which was only slightly and variably improved by doubling this concentration. Potassium chloride at 50 mg./100 ml. supplies to the medium *c.* 26 mg. potassium, which with the *c.* 3 mg. supplied as phosphate and *c.* 14 mg. used as hydroxide in adjusting the pH value in these experiments brings the total required concentration of potassium to *c.* 43 mg./100 ml. In some experiments there were indications of a requirement for a constant ratio between sodium and potassium ions. In the narrow concentration ranges in which this occurred the obtainable differences in growth were, however, within the variation which normally occurred from experiment to experiment. It was therefore not considered worth while to pursue this line of investigation. The growth of isolate A was unaffected by the addition of potassium chloride to media lacking it.

Since preliminary experiments had indicated that magnesium and calcium could replace each other to a certain extent, experiments to determine the optimal concentrations of magnesium (weighed and used as $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) and calcium (weighed as CaCO_3 and used as hydrochloric acid solution) ions were designed so as to test this point as well. No growth of isolates M and P occurred at 1·0 mg. Ca/100 ml. or less, or at 0·1 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ /100 ml. Isolate A grew,

though poorly, at 0.05 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}/100$ ml. (in the presence of 8.0 mg. $\text{Ca}/100$ ml.), and in the absence of added calcium (in presence of 0.4 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}/100$ ml.). Maximal growth of all strains occurred at 8–10 mg. $\text{Ca}/100$ ml. and 0.5 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}/100$ ml. The calcium carbonate of the stock medium supplied 8.0 mg./100 ml. of calcium ions. The data of these experiments are unsuitable for graphic presentation for the following reasons: the concentration of inorganic constituents of the medium affects rate as well as total amount of growth, and maximum growth is followed rapidly by

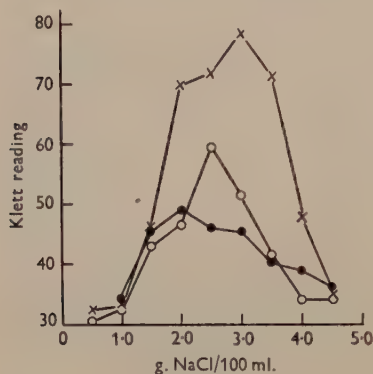


Fig. 1

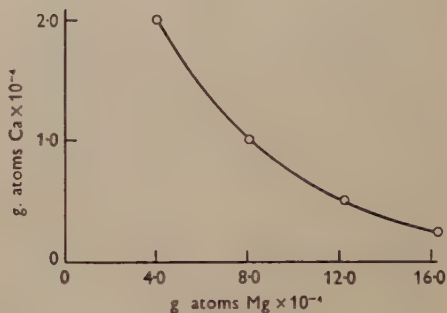


Fig. 2

Fig. 1. Growth response of isolates A (●), M (×), and P (○) to increasing concentrations of NaCl after incubation at 20° for 5, 4 and 5 days. Klett readings below 36 indicate no growth.

Fig. 2. Equivalents of Mg and Ca ions/100 ml. allowing growth of isolate A to Klett reading 70–71 in 9 days.

autolysis. Since these experiments were read quantitatively only after 7 or 9 days of incubation, in order to follow the effects of the lower concentrations, flasks containing more nearly optimal concentrations were already beginning to undergo autolysis. The degree of replacement of magnesium and calcium by each other can, however, be seen in Fig. 2, in which the combinations of calcium and magnesium are plotted which had just resulted in growth to Klett reading 70–71 of isolate A at the conclusion of the experiment (9 days). It will be noted that the replacement equivalence depends upon which of the variables is more limiting. Some of the replacement effect is undoubtedly due to the mass action of one or the other ion in displacing the other from the chelating agents present in the medium. The strongest of these chelating agents however (EDTA), is present at a concentration of only $3.05 \times 10^{-4}\text{M}$ which is too little to bind all of the eligible metallic ions present. It was assumed that sulphate was not limiting at the lowest concentration of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ which permitted growth, since other metals were supplied as sulphates. In an experiment designed to test this assumption, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was replaced by an equivalent amount of magnesium as chloride and sodium sulphate. The use of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ resulted in the formation of a precipitate and in poor or no growth whether or not sodium sulphate was present.

When sodium chloride, potassium chloride, potassium phosphate, magnesium sulphate, and calcium carbonate were supplied in the amounts necessary for maximal growth, the addition of other inorganic nutrients (in the absence of EDTA) was superfluous. Such a medium develops the precipitate common to artificial sea waters. This precipitate is undesirable: it is a source of uncontrolled variation in the composition of the medium and in measuring growth spectrophotometrically. The precipitate does not form, and growth is markedly improved, when EDTA and the micronutrient elements are added as indicated in Table 1. The formula given was designed for isolate M, but gives good growth of isolates A and P. Increase in no single micronutrient improved the growth of isolate M. The inclusion of gelatin hydrolysate (or amino acids) in this medium, as well as the large amounts of salts required in any marine medium, make it unsuitable for the demonstration of micronutrient requirements. Nevertheless, in experiments with all three isolates in which the micronutrients were completely omitted from the stock medium, there was no growth. When the micronutrient elements were omitted singly, there was little or no growth in the absence of iron, a marked decrease in rate and amount of growth in the absence of manganese, and with isolate P a marked decrease in amount of growth in the absence of zinc.

Vitamin requirements

All isolates failed to survive continued subculture in media lacking thiamine. Fig. 3 illustrates the response of isolate M to thiamine concentration after two passages through thiamineless media. The basal medium for this experiment contained an amino acid mixture instead of gelatin hydrolysate and only one-half the amount of agar normally used. The addition of a mixture of water-soluble vitamins (B_{12} , *p*-aminobenzoic acid, nicotinic acid, calcium pantothenate, pyridoxamine.HCl, biotin, folic acid) to media containing thiamine did not further improve growth of any isolate.

Carbon and nitrogen requirements

In preliminary experiments all isolates grew well with gelatin hydrolysate as carbon and nitrogen source but gave little indication of utilizing for growth any of the commonly used non-nitrogenous substrates, whether nitrogen was supplied as ammonium salts or by the addition of small amounts of amino acids. The following amino acids were therefore substituted singly (at 0.05 g./100 ml.) for the gelatin hydrolysate of the stock medium: DL-alanine, L-arginine.HCl, (K) L-aspartic acid, (Na) L-glutamic acid, glycine, L-histidine.2HCl, DL-isoleucine, L-leucine, L-lysine.HCl, DL-methionine, DL-phenylalanine, L-proline, DL-serine, DL-threonine, DL-tryptophan, L-tyrosine and DL-valine. The use of large inocula permitted growth on (in order of decreasing effectiveness): isolate M—L-leucine, DL-alanine, L-proline, L-glutamic acid; isolate A—L-glutamic acid, L-aspartic acid, DL-alanine, L-proline; isolate P—L-glutamic acid, L-proline, DL-alanine, L-arginine.HCl.

L-Histidine, DL-isoleucine, DL-methionine, DL-phenylalanine, L-tyrosine, and DL-valine in substrate concentration decreased or prevented growth of all isolates even in the presence of gelatin hydrolysate at 0.01 g./100 ml. The toxicity of isoleucine and valine, at concentrations of 1.0 mg./100 ml. was annulled by combining them, as for certain mutants of *Escherichia coli* (Davis, 1950) and *Neurospora* sp. (Bonner, 1946). Glycine and DL-tryptophan were toxic for isolates A and M; L-aspartic acid for isolate P; and DL-threonine for isolate A.

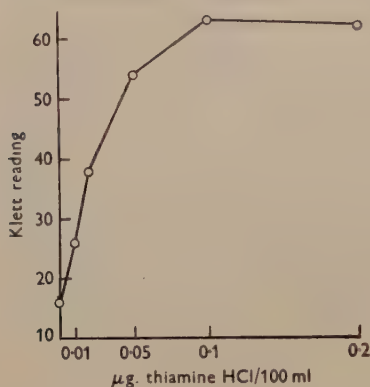


Fig. 3

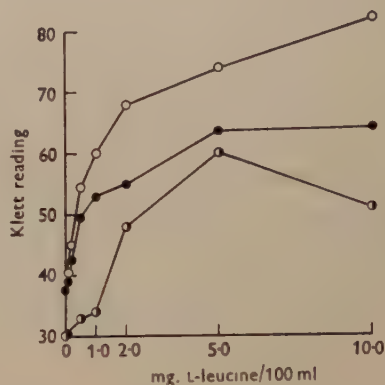


Fig. 4

Fig. 3. Growth response of isolate M to increasing concentrations of thiamine. HCl after incubation for 7 days at 20°. No growth occurred in the absence of thiamine.

Fig. 4. Growth response of isolate M to increasing concentrations of L-leucine after incubation at 20° for 7 days. Substrates were L-proline (●), DL-alanine (●), and NaHglutamate (○), 0.05 g./100 ml. No growth occurred in the absence of leucine.

Each isolate was then serially subcultured in media containing (separately) the amino acids on which it had grown in this experiment. Subcultures were continued until growth ceased or for three to six transfers. Growth of isolate A reached a stable level on the second transfer: NaHglutamate and L-aspartic acid continued to be well utilized as substrates, though gelatin hydrolysate gave better growth at the same concentration, while DL-alanine and L-proline gave continuously culturable, but very poor, growth. Growth on L-aspartic acid was not significantly improved by the addition of 0.5–5.0 mg./100 ml. of any other single amino acid. Isolate P similarly continued to grow excellently on NaHglutamate, and as well or better on L-proline and DL-alanine as on an equal concentration of gelatin hydrolysate. Growth on L-arginine ceased on the second transfer. Growth of isolate P on DL-alanine or L-proline was not significantly improved by the addition of any other single amino acid at 0.5–5.0 mg./100 ml.

Isolate M, however, ceased to grow on the second transfer except on L-leucine, which supported very feeble growth. Although unsuitable as substrate, L-leucine (5.0 mg./100 ml.) alone of the 17 amino acids tested, restored growth of isolate M when DL-alanine, L-proline, or NaHglutamate was used

as substrate. These cultures were carried through three transfers on each substrate in the presence of L-leucine. Growth was approximately equal, in all cultures, to that supported by a concentration of gelatin hydrolysate equal to that of the substrate amino acid (0.05 g./100 ml.). The response of isolate M to L-leucine concentration after a single passage through leucineless media is seen in Fig. 4. Growth tended to be rather sparse and uneven in the experiments illustrated in Fig. 4 because of the sparse inoculum used. L-Leucine alone was inhibitory at 5.0 mg./100 ml.; in the presence of other amino acids 10 or 20 mg./100 ml. slowed growth.

The experiments just outlined clearly indicate that isolates A and P, although only able to utilize amino acids as substrates for growth, do not require specific amino acids as growth factors. Glucose, ethanol, mannitol, Na lactate, Na acetate ($3\text{H}_2\text{O}$), glycerol, Na succinate ($6\text{H}_2\text{O}$), Na fumarate, Na malate, and Na citrate ($5\frac{1}{2}\text{H}_2\text{O}$) at 0.1 g./100 ml. failed to support significant growth of isolates A and P in the presence of gelatin hydrolysate at 0.01 g./100 ml.

Since isolate M is auxotrophic for L-leucine, further tests were made of a variety of compounds as substrates in the presence of L-leucine (5.0 mg./100 ml.). Of amino acids not previously utilized as substrates, L-arginine.HCl, (K) L-aspartic acid, and DL-phenylalanine supported equally modest growth at 0.05 g./100 ml. Soluble starch, sucrose, lactose, glucose, fructose, arabinose, xylose, ethanol, glycerol, mannitol, Na acetate ($3\text{H}_2\text{O}$), Na lactate, Na fumarate, Na citrate ($5\frac{1}{2}\text{H}_2\text{O}$), Na malate, Na succinate ($6\text{H}_2\text{O}$), and Na gluconate were also tested at 0.05 g./100 ml. Sugars were sterilized by filtration and added, as was ethanol, aseptically to sterile basal medium. Of these addenda, only glycerol supported significantly more growth than the basal medium alone. This poor and variable growth was not improved by the addition of Krebs cycle acids (succinic, fumaric, malic, or citric) to a medium in which the gelatin hydrolysate of Table 1 was replaced with glycerol (0.1 g./100 ml.) and gelatin hydrolysate (0.01 g./100 ml) at either pH 7.0 or pH 7.8. It is possible that the metal-binding properties of glycerol, rather than its energy content, are responsible for its effect on growth.

DISCUSSION

The ocean as an environment for micro-organisms has received little attention in comparison with fresh-water and terrestrial environments. The inorganic composition of the ocean, and its essential constancy, is well established. A series of studies (see Harvey, 1945, 1949) has indicated that the growth of phytoplankton in sea water is frequently limited by insufficient phosphate, nitrate, silicate, iron and manganese. But the relationship between those elements which are not limiting in sea water and the growth of marine micro-organisms remains almost unknown. What are the elements in sea water which define the stenohaline niche? Cultural studies of marine micro-organisms have usually been carried out in sea water enriched with the known limiting elements (and perhaps a pinch of mud) or in artificial sea waters made up with more

emphasis on the composition of natural sea water than the requirements of the micro-organisms concerned. The pioneering studies of Hutner (1948), using the inshore diatom *Nitzschia closterium*, indicated that considerable liberties could be taken with the composition of sea water in preparing a medium for this organism. In Table 2 the comparison is made between the concentrations of the major inorganic compounds of sea water (from Sverdrup, Johnson & Fleming, 1942), Hutner's medium for *Nitzschia* sp., and the labyrinthula medium

Table 2. *A comparison of the major constituents of sea water and two media for marine organisms*

	Sea water (Cl = 19.00 ‰)		Labyrinthula medium		Nitzschia medium	
	mg./100 ml.	mg.-atoms/l.	mg./100 ml.	mg.-atoms/l.	mg./100 ml.	mg.-atoms/l.
Na ⁺	1055.61	470.15	984	428	86.9	37.8
Mg ⁺⁺	127.20	53.57	49.3	20.3	24.7	10.15
Ca ⁺⁺	40.01	10.24	8.0	2.0	3.5	0.875
K ⁺	38.00	9.96	55.3	14.1	7.56	2.3
Sr ⁺⁺	1.33	0.15	0.0	0.0	0.0	0.0
Cl ⁻	1897.99	548.30	1567.7	441.4	121.4*	34.2*
SO ₄ ⁼	264.86	38.24	170.8	20.5	97.4*	10.15*
HCO ₃ ⁻	13.97	2.34	6.25†	1.04†	not det.	not det.
Br ⁻	6.46	0.83	0.0	0.0	0.0	0.0
H ₃ BO ₃	2.60	0.43	0.114	0.00324	0.286	0.00462
F ⁻	0.13	0.07	0.0	0.0	0.0	0.0

* The chloride and sulphate added as acid used to dissolve metals are not included in these calculations.

† Calculated from added ammonium carbonate. The bicarbonate content of the medium as used was not determined.

reported here. It is evident that the more exacting forms—the labyrinthulas—require not only a relatively narrow range of sodium chloride concentrations but also concentrations of magnesium, calcium and potassium which are of the same order of magnitude as the concentrations of these elements in the ocean. Using such requirements as criteria of stenohalinity, isolates M and P are most stenohaline and would be expected to occur primarily in open ocean or inshore areas of little land drainage. Isolate A, although its range of sodium chloride tolerance is the same as that of isolates M and P, is less stenohaline; the optimum sodium chloride is lower, magnesium and calcium requirements are less rigid, and the high potassium requirement is absent. This strain would be expected to predominate in estuarine habitats. The ecological studies which would provide a test of the applicability of such laboratory findings have not, however, been made. Moreover, the unpublished data of Dr S. W. Watson indicate that both isolates A and M grew as well on serum sea water agar made with 50 % (v/v) sea water, as when 100 % sea water was used.

Since the labyrinthulas are heterotrophic, one cannot consider the small and ill-defined organic content of the ocean to be the source of their organic requirements; thiamine, steroids and amino acids are readily available from host plants. The host plants serve other, less obvious, functions as well,

providing the physical support necessary for motility of *Labyrinthula* spp. and providing oxygen. The isolates of *Labyrinthula* used in these studies were so highly aerobic that they could surely not compete for food with the bacterial flora of the ocean except in association with actively photosynthesizing organisms. They were unable, for example, to flourish under a 7–8 mm. stationary layer of liquid medium.

It is a pleasure to acknowledge my indebtedness to Dr S. W. Watson for the isolates used in this study and for permission to use his unpublished data, to Dr S. H. Hutner of Haskins Laboratories, where these studies were begun, and to Mr S. E. Mills, for expert technical assistance. These studies were aided by a grant from the Eugene Higgins Fund and by a contract between the Office of Naval Research, Department of the Navy, and Yale University, NR 135–241.

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[The Activity of Steroids as Growth Factors for a *Labyrinthula* sp.]

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SUMMARY: Further investigation of the specificity of the steroid growth factor requirement of *Labyrinthula vitellina* var. *pacifica* has provided a detailed description of the structural requirements for activity. Active compounds include sterols and products of their autoxidation; the activity of the sterols is not, however, due to autoxidation during the assay period. The data obtained circumscribe the characteristics of the essential metabolite represented by this requirement but do not identify it.

Labyrinthula vitellina var. *pacifica* shows a steroid growth factor requirement which can be satisfied by cholesterol, Δ^4 -cholestenone, fucosterol, or β -sitosterol (Vishniac & Watson, 1953) but not by any of twenty other steroids or by the non-steroids which annul fatty acid toxicity. Such a requirement represents inability to synthesize an essential metabolite. This paper describes the results of testing additional steroids as growth factors, in order to further circumscribe the possible characteristics of the metabolite.

METHODS

The following modifications of the assay method used by Vishniac & Watson (1953) were found to improve the sensitivity and reliability of the assay for growth factor activity. (a) The basal medium of Vishniac (1955) is now used. (b) Steroid samples are added as freshly prepared ethanolic solutions, serially diluted so that 1 drop of solution/10 ml. medium is used. Addition is made after the medium has been distributed and sterilized. (c) Inoculation is made directly from stock cultures, using one drop of a mature but recent culture into 10 ml. of medium. The few compounds which were tested by earlier methods are indicated by an asterisk * when results are given. After 7 days incubation at 20-21°, assays were read, in a Klett-Summerson photoelectric colorimeter with no. 42 filter.

Quantitative tests for cholesterol, 7 α - and β -hydroxycholesterols, and cholestane-3 β , 5 α , 6 β -triol were performed, using appropriate modifications of the method of Moore & Baumann (1952) and Idler & Baumann (1953). The last compound does not form a digitonide and in its free form is a weak chromogen, reaching maximum colour development at 620 m μ . in 7 min. A much higher peak is present at 420 m μ . which continues to develop sufficiently slowly for use after 40 min. The 7 α - and β -hydroxycholesterols were also assayed by using the Lifschutz reagent according to Bergstrom & Wintersteiner (1942a); 7-ketocholesterol was determined by optical density at 240 m μ . in absolute ethanol (Wintersteiner & Bergstrom, 1941).

The steroid preparations used were: cholesterol (Pfanstiehl Chemical Co.); cholestenone (Matheson, Coleman and Bell Inc.); cholestane, clionasteryl acetate, poriferasterol, neospongosteryl acetate, palysterol, haliclonasterol, α -stellasterol, β -stellasterol, spinasterol, campesterol (gifts of Dr W. Bergmann, Department of Chemistry, Yale University); β -sitosterol (L. Light and Co. Ltd.); stigmasterol (gift of Dr W. van Wagtendonk, Department of Zoology, Indiana University); ergostane, ergosterol, $\Delta^{14(15)}$ -ergosterol, Δ^7 -ergosterol (gifts of Dr Seymour Bernstein, Lederle Laboratories); $\Delta^8(14)$ -ergosterol, α -dihydroergosterol, 7-ketocholesterol, 4(β)-hydroxycholesterol (gifts of Dr Harris Rosenkrantz, The Worcester Foundation for Experimental Biology); squalene, pregnenolone, dehydroisoandrosterone (gifts of Dr K. Savard, The Worcester Foundation for Experimental Biology); $\Delta^8(14)$ -cholestenyl benzoate, 7-ketocholesteryl acetate, Δ^4 -cholestenol, 3(β), 7(β)-dihydroxycholestanyl acetate, 7(α)-hydroxycholesteryl-3-benzoate (gifts of Dr D. R. Idler, Fisheries Research Board of Canada); 7-dehydrocholesteryl acetate (gift of Dr R. G. Langdon, Department of Biochemistry, University of Chicago); cerebrosterol, cerebrostenolone, 24-ketocholesterol (gifts of Dr A. Ercoli, Vismara Terapeutici); Δ^4 -cholestadiene-3-one (gift of Dr J. Berlin, Syntex, S.A.); Windaus keto acid (gift of Dr T. Stadtman, National Heart Institute); lathosterol, 'lipid diol', Butenandt acid, Butenandt ketone, Diels acid, cholestane-3, 6-dione, cholestane-5 α -ol-3, 6-dione, cholestane-3 β , 5 α , 6 β -triol, Δ^5 -cholestene-3-one, Δ^4 -cholestene-6 β -ol-3-one, Δ^4 -cholestene-3, 6-dione (gifts of Dr L. F. Fieser, Department of Chemistry, Harvard University); β -hydroxy- β -methyl glutaric acid, β -methyl glutanoic acid (gifts of Dr F. Dituri, Department of Physiological Chemistry, University of Pennsylvania); 7(α)-hydroxycholesterol and Δ^6 -cholestenyl acetate (gifts of Dr O. Wintersteiner, The Squibb Institute for Medical Research). Compounds received as esters were saponified before use. Δ^3 ,5-cholestadiene-7-one was made from 7-ketocholesteryl acetate (Bergstrom & Wintersteiner, 1941).

Many of these preparations were available in such minute quantities that they could only be assayed as received. When sample size permitted, preparations were recrystallized several times from methanol and acetone. Recrystallization was particularly helpful in removing the toxic contaminants usually found in aged or off-colour preparations. When possible, the melting points of preparations were taken in a Dow-Corning No. 200 Fluid bath. Melting points given are uncorrected. Assays were run with 1, 2, 5, 10, 20 and 50 μ g. steroid/10 ml. of medium. A duplicate series with 50 μ g. cholesterol/10 ml. of medium, added before sterilization, was used as toxicity control.

RESULTS

The difficulties encountered because of small sample sizes and the sensitivity of *Labyrinthula vitellina* var. *pacifica* to toxic contaminants in many of the samples made it possible to obtain trustworthy response curves covering the complete concentration range with only a few steroids. The activity of representative steroids for which good samples were available is shown in Fig. 1.

Of those related steroids which differed only in the tail portion of the cholesterol molecule in:

(a) *Length*. Pregnenolone (m.p. 187–190°) was, as progesterone, completely inhibitory; dehydroisoandrosterone (m.p. 150°) inactive.

(b) *Substituents at C24*. Campesterol* (C24a methyl), clionasterol (C24a ethyl), β -sitosterol (C24b ethyl) (m.p. 137°), α -stellasterol (C24a methyl), and $\Delta^{8(14)}$ -ergosterol (C24b methyl) (m.p. 131°) were active. The recrystallized preparation of β -sitosterol used was as active in the lower range of concentra-

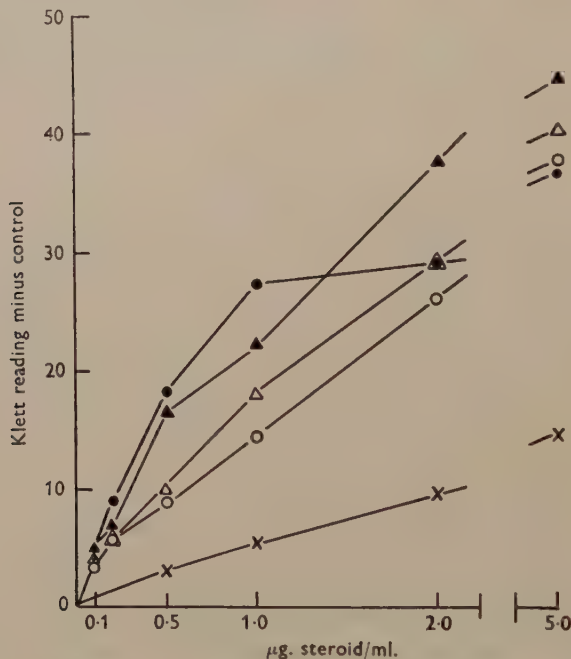


Fig. 1. Growth response of *Labyrinthula vitellina* var. *pacifica* to varying concentrations of steroids. Controls showed no growth. x = stigmasterol; o = cholesterol; Δ = $\Delta^5, 14$ -ergosterol; \blacktriangle = cholestane-3 β , 5 α , 6 β -triol; \bullet = cholestenone.

tions (10 μ g./ml. and below) as the recrystallized cholesterol (m.p. 148.5–149°) for which data are given in Fig. 1, but failed to keep pace at higher concentrations. The other Δ^5 -stenols used were considerably less active than cholesterol at all concentrations, presumably because they were obviously inferior preparations, although growth in the presence of added cholesterol was not significantly affected. The same was true of palysterol* and haliclasterol*, of incompletely known structure. Recrystallized cerebrosterol (Δ^5 -cholestene-3 β , 24 ξ' -diol; m.p. 175–175.5°) and 24-ketocholesterol (m.p. 135°) were inactive and were toxic at 5 μ g./10 ml. or more. Recrystallized cerebrostenolone (Δ^4 -cholestene-3, 24-dione; m.p. 135°) was inactive and toxic at 20 μ g./10 ml.

(c) *Unsaturation at C22 (23)*. Recrystallized poriferasterol (m.p. 132°) and stigmasterol (m.p. 137°) showed, as did brassicasterol previously, some activity.

Since the stigmasterol preparation appeared good and had full activity for a *Paramecium* sp. (as tested by Dr W. J. van Wagtenonk), this activity is now considered to be real (see Fig. 1).

Of those related steroids which differed in the hydrogenation of the ring portion of the cholesterol molecule which were:

(a) *Saturated*. Cholestane*, ergostane*, and ergostanol*, as cholestanol and coprosterol previously, were inactive. Neospongosterol showed traces of activity (20 μ g. giving the growth produced by 1 μ g. of the most active compound) presumed to be due to sterol impurities.

(b) *Singly unsaturated*. Δ^4 -Cholestenol (allocholesterol; m.p. 129.5°) was only slightly more active than stigmasterol. This activity could be the result of non-biological cholesterol formation during the assay period. Recrystallized Δ^4 -cholestenone was one of the most active steroids (see Fig. 1); Δ^5 -cholestene-3-one had only about the activity of cholesterol; Δ^6 -cholestenol (m.p. 134.5–136°) was presumed inactive since growth occurred only with 50 μ g./10 ml. and even then merely equalled the growth produced by 2 μ g. of the most active steroids. Δ^7 -Ergostenol, 5-dihydroergosterol, and spinasterol were inactive; the slight activity of lathosterol (Δ^7 -cholestenol) was halved on recrystallization (so that maximum growth, at 10 μ g. was equal to the growth produced by 1 μ g. of the most active compound). I conclude that Δ^7 -stenols are not active. Recrystallized $\Delta^{8(14)}$ -ergostenol (m.p. 131°) was slightly more active than cholesterol (see Fig. 1); $\Delta^{8(14)}$ -cholestenol (m.p. 120–121°), and recrystallized α -stellasterol were equally active at 5 μ g./10 ml. or below. $\Delta^{14(15)}$ -ergostenol* and β -stellasterol* were inactive.

(c) *Dienes*. 7-Dehydrocholesterol (sintered at 128°) was about as active as cholesterol. The absence of a good melting point makes the significance of this result rather doubtful, although ergosterol has previously shown some very slight, variable, activity. Both preparations had to be recrystallized to free them of toxic material. Both compounds are known to deteriorate during recrystallization (Huber, Ewing & Kriger, 1945).

The results of testing the products of autoxidation of cholesterol suggested that the naturally occurring sterols might be active by virtue of autoxidation occurring during the 7-day incubation period of the assays. Cholesterol undergoes rapid autoxidation in colloidal aqueous solution (Bergstrom & Wintersteiner, 1942*b*) yielding 7 α -hydroxycholesterol, 7-keto-cholesterol, $\Delta^{3,5}$ -cholestadiene-7-one (Wintersteiner & Bergstrom, 1941; Bergstrom & Wintersteiner, 1941) and cholestane-3 β , 5 α , 6 β -triol (Mosbach, Nierenberg & Kendall, 1953). At lower temperatures (37°) 7-ketocholesterol predominates (Bergstrom & Wintersteiner, 1942*a*). Recrystallized 7-ketocholesterol (m.p. 172–172.5°) gave an activity curve almost superimposable upon that of $\Delta^{8(14)}$ -ergostenol (see Fig. 1). Cholestane-3 β , 5 α , 6 β -triol (m.p. 237–238°) was even more active (see Fig. 1). 7 α -Hydroxycholesterol (m.p. 150–151°) was as active (at low concentrations only) as cholestenone.

Since autoxidation is catalysed and inhibited by various metallic ions (Bergstrom & Wintersteiner, 1942*b*), it was impossible to predict whether it would occur in the complex medium used. Lots (250 ml.) of medium prepared

as for assay experiments were, therefore, distributed in Fernbach flasks to form layers 7–8 mm. deep. The flasks were then plugged, autoclaved, and cooled to 20°. In separate experiments 1.25 and 7.5 ml. portions of an 0.2% (w/v) solution of freshly recrystallized cholesterol in 95% (w/v) ethanol in water were added to each lot of medium. After 7 days incubation at 20–21° the flask contents were extracted continuously with ether under a stream of alkaline-pyrogallol-washed nitrogen for 8 hr., or, when these precautions proved unnecessary, shaken with four 70 to 30 ml. portions of ether. In the latter case, backwashing each portion of ether with 10 ml. distilled water was necessary to break the emulsion stabilized by the agar of the medium. The ether extract was then evaporated under vacuum and dried in a vacuum desiccator over silica gel. Although difficulty was experienced in recovering cholesterol from the less concentrated preparation by the methods outlined, no 7 min. excess was found in the Moore-Baumann test, no Lifschutz reaction was given by a sample equal to 2/10 of the residue of two flasks, and no peak appeared at 240 m μ . From the more concentrated preparation 99% of the cholesterol was recovered. There were again no indications of the presence of any fast Moore-Baumann steroids, Lifschutz-reacting steroids, or substances giving peaks in the ultraviolet. Autoxidation does not occur under the conditions of these experiments.

The results of testing other products of the mild oxidation of cholesterol were as follows:

(a) *Cholestanes*. Cholestane-3, 6-dione (m.p. 170–171°) gave an activity curve almost superimposable upon that of cholestane-triol (see Fig. 1). Recrystallized cholestane-3 β , 7 β -diol (m.p. 150–153°) was inactive and showed increasing toxicity with increasing concentration. Cholestane-5 α -ol-3, 6-dione (m.p. 244–245°), used as an acetone solution, was inactive. Acetone was not responsible for absence of activity.

(b) *Cholestenes*. The activity of Δ^4 and Δ^5 -cholestenone has been mentioned earlier. Δ^4 -Cholestene-6 β -ol-3-one (m.p. 187–188°) was about equally active as cholestane-triol; Δ^4 -cholestene-3, 6-dione (m.p. 123–124°) as cholesterol. Recrystallized 4 β -hydroxycholesterol (m.p. 177°) was inactive and slightly inhibitory at the higher concentrations used.

(c) *Cholestadienes*. $\Delta^{3,5}$ -Cholestadiene-7-one (m.p. 112°) was inactive. $\Delta^{4,6}$ -cholestadiene-3-one (m.p. 75–76°) was as active as $\Delta^{8(14)}$ -ergosterol at 5 μ g./10 ml. and below, but increasing concentrations had no greater effect on growth.

Products of oxidation so violent as to open or shorten a ring were inactive and more or less toxic: Windaus keto acid* was completely inhibitory; Diels acid and Butenandt ketone inhibitory at 10 μ g. or more/10 ml.; Butenandt acid at 20 μ g. or more/10 ml.

Cholesterol from natural sources contains steroid contaminants in amounts too small to account for its activity. The possibility that activity was due to a non-steroid contaminant, while rather far-fetched in view of the indirectly biological provenance of many active compounds, led us to examine the 'lipid diol' (CH₃.CH₂.CH(CH₃).CH₂.(CH₂)₁₅.CHOH.CH₂OH) isolated by Dr L. F. Fieser from cholesterol; it was inactive.

The possibility that the effective block in biosynthesis of the steroid metabolite required by these *Labyrinthula* isolates might lie in an early portion of the pathway led us to examine non-steroid precursors of cholesterol. Squalene* a precursor of cholesterol (Langdon & Bloch, 1952, 1953*a*), was inactive and became toxic at 25 μ g./10 ml. β -Hydroxy- β -methylglutaric acid (Rabinowitz & Gurin, 1954*a*) and β -methylglutaconic acid (Rabinowitz & Gurin, 1954*b*) have also been suggested as precursors of cholesterol; neither was active.

DISCUSSION

The data of Vishniac & Watson (1953) indicated that the steroid growth factor requirement of *Labyrinthula vitellina* var. *pacifica* could be satisfied by cholesterol or derivatives formed by the addition of a C24b ethyl group or C24 vinyl group or by mild oxidation to Δ^4 -cholestenone. Activity was abolished by changing the orientation of, or esterifying, the C3 hydroxyl, by saturating the double bond, or by introducing C22(23) unsaturation (or making more radical alterations) in the tail of the cholesterol molecule. The experiments reported above make it possible to specify further that:

- (1) Only steroids with a 3β -OH or 3-keto group are active.
- (2) Only C27, 28 and 29 steroids are active.
- (3) Of these, both of the possible C24 methyl and ethyl epimers derived from active cholestenols are active. Cholesterol derivatives oxidized at C24 are inactive.
- (4) If no other oxygen function (than as (1)) is present, only steroids unsaturated at C4(5), or C5(6), or C8(14) are active. Ring-saturated steroids, or those unsaturated only at C6(7), C7(8), or C14(15), are inactive.
- (5) $\Delta^{5,7}$ (?) and $\Delta^{4,6}$ -Dienes are active. $\Delta^{5,22}$ -Dienes have low activity.
- (6) Steroids with more than one oxygen function on the rings need not be unsaturated: cholestane- 3β , 5 α , 6 β -triol and cholestane-3,6-dione are active. However, cholestane- 3β , 7 β -diol and cholestane-5 α -ol-3, 6-dione are inactive.
- (7) The activity of cholesterol is retained when 7-keto or 7 α -OH functions are added, but not when a 4 β -OH is added. Δ^4 -Cholestenone activity is likewise retained when 6-keto or 6 β -OH groups are added.

It seems highly unlikely that the apparent activity of these steroids (excepting allocholesterol and 7-dehydrocholesterol) is due to chemical contamination or to non-biological changes occurring during the assay period. Any active compound must then belong to a biochemical family consisting of (a) an essential metabolite, (b) natural precursors in its biosynthesis, and (c) compounds biologically convertible into the metabolite, but not normal precursors in biosynthesis. This family probably arises after the cyclization of squalene and certainly does not include any of the short-tailed descendants of cholesterol (Zabin & Barker, 1953; Siperstein, Harold, Chaikoff & Dauben, 1954). Singly unsaturated steroids are active only when they derive their unsaturation from squalene, folded as postulated by Bloch (Langdon & Bloch, 1953*b*).

Since the relative activities of useful steroids constitute insufficient evidence

for assigning any one of them the role of essential metabolite, other lines of inquiry must be adopted. Isolation of steroids from related *Labyrinthula* spp. which do not require a steroid as nutrient is being attempted.

Comparison of the specificity patterns of other organisms with similar requirements yields a picture capable of several interpretations; such a comparison is made in Table 1. One other micro-organism, *Saccharomyces cerevisiae* SC-1 (DCL), has been reported to require a steroid growth factor on a defined medium when grown anaerobically (Andreasen & Stier, 1953); no study of specificity was made and cholesterol, ergosterol, or anti-stiffness factor (stigmasterol) satisfied the requirement.

Table 1. *The specificity of the steroid growth factor requirements of micro-organisms*

	<i>Trichomonas columbae</i> (Cailleau, 1937)	<i>Paramecium aurelia</i> (Conner & van Wagtendonk, 1955)	<i>Labyrinthula</i> (present work)	PPLO* (Edward & Fitzgerald, 1951)
3β-OH-Steranes:				
Cholesterol	+	0	0	+
Coprosterol	0	0	0	0
Ergosterol	+	0	0	.
Δ^5-Ene-3-ols:				
Cholesterol	+	0	+	+
β -Sitosterol	0 (?)	+	+	.
Clionasterol	+	+	+	.
Fucosterol	.	+	+	.
$\Delta^{5,22}$-Diene-3-ols:				
Stigmasterol	0	+	\pm	+
Δ^8 (14)-Ene-3-ols:				
α -Ergosterol	+	0	+	.
$\Delta^{5,7}$-Diene-3-ols:				
7-Dehydrocholesterol	+	.	+	.
22-Dihydrogosterol	+	.	.	.
$\Delta^{5,7,22}$-Triene-3-ols:				
Ergosterol	\pm	0	0 (?)	0
Δ^5-Ene-diols:				
Δ^5 -Cholestene-3, 4-diol	+	.	0	.
Oxidation products:				
Δ^5 -Cholestenone	0	.	+	.
Δ^4 , 22-Stigmastadienone	.	+	.	.
Cholestane-3, 6-dione	0	.	+	.

0=no utilization; \pm =some utilization; +=utilization of steroids as growth factors; . =not tested; (?) = validity of the datum is questionable; *PPLO=pleuropneumonia-like organisms.

It is evident from Table 1 that while no single steroid is active for all of these micro-organisms, further investigation might easily indicate a common factor. The differences in pattern are, however, sufficiently great to make tenable the hypothesis that no single essential metabolite is represented by these requirements. The most conspicuous differences are the use of saturated 3 β -hydroxy sterols by *Trichomonas* spp. and pleuropneumonia-like organisms and the use of certain oxidation products of active sterols by *Paramecium aurelia* and *Labyrinthula vitellina* var. *pacifica*. These differences, and the multiplicity of roles assumed by steroids in mammals suggest that different

metabolites with different roles may be represented. Another possibility, that a single biochemical role might be assumed by different sterols in different organisms is suggested by the use of C27 steroids by the *Labyrinthula* sp., while *Paramecium aurelia* uses only C28 or C29 steroids. The sterols of various taxonomic groups are usually characteristic and significant in biochemical evolution (Bergmann, 1949, 1953; Heilbron, 1942). A non-characteristic sterol might still be active, either directly by virtue of chemical homology or indirectly by conversion, in the same reactions as was the sterol which occurred naturally in a particular group. Studies on the metabolism of labelled cholestanol, cholesterol, and cholestenone by the appropriate micro-organisms should at least clarify these questions.

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The Structure of *Saccharomyces carlsbergensis* and *S. cerevisiae* as Determined by Ultra-thin Sectioning Methods and Electron Microscopy

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SUMMARY: Cells of *Saccharomyces carlsbergensis* and *S. cerevisiae* were fixed with osmic acid, embedded in plastic, sectioned at one-twentieth of a micron, and then observed in the electron microscope. The pictures obtained gave confirmation of the double membrane nature of the yeast cell wall reported by earlier workers such as Northcote & Horne (1952), and gave some indication of the presence of additional very thin membranes in the cell wall. It was suggested that the two cell wall membranes might be the two osmotic barriers observed to be present in the yeast cell wall by Conway & Downey (1950). No structure was seen which could be identified as the cytoplasmic membrane.

Guilliermond (1920) wrote that at least two investigators, H. Will in 1896 and O. Casagrandi in 1897, had reported that yeast cell walls consisted of two distinct and separate membranes. These early workers reported that the two membranes were seen in older cultures after treating the cells with hydrochloric acid, and that osmic acid was especially good for making the membranes visible. Guilliermond also wrote, and it is common knowledge, that the yeast cell wall develops from a thin membrane into a rather thick membrane as the cell ages. However, the evidence for the double membraned cell wall seems not to have been accepted by most writers of text-books since they generally have failed to mention this point. Impressive evidence for the double cell-wall membranes was recently presented by Northcote & Horne (1952). They obtained preparations of the yeast cell membrane, free from cytoplasm and whole cells, by mechanically rupturing the cells and following this by differential centrifugation. They state that '...when the preparations were treated with 2N-HCl...it could be seen that the cell envelope consisted of at least two membranes'. They also presented an electron microscope picture clearly showing these two membranes in a cell wall fragment of *Saccharomyces cerevisiae*. The demonstration of two membranes in the yeast cell wall is of importance since they might be expected to play an important role in the behaviour of the cell wall as an osmotic barrier. Conway & Downey (1950), also working with *S. cerevisiae*, reported the presence of two osmotic barriers in the cell wall. The outer barrier was reported to be permeable by acetic, propionic, succinic, and glyceric acids, but not by inulin or peptone. The inner barrier was reported as being permeable by acetic and propionic acids, but not by succinic or glyceric acids. The area into which the succinic and glyceric acids penetrated was shown to be the outer 10-11 % of the total cell volume, and was assumed to represent the cell-wall area.

The present paper presents the results of the application of thin sectioning techniques in an attempt to study the structure of the yeast cell, and in particular the structure of the yeast cell wall.

METHODS

Cultures of *Saccharomyces carlsbergensis* and *S. cerevisiae* were grown for 72 hr. in glucose yeast extract broth at 30° on a shaking machine. The cells were then removed by centrifugation and washed twice with distilled water. The fixing and plastic-embedding procedures were those described for bacteria by Chapman & Hillier (1953) except that in some instances the osmic acid fixation was omitted. Sectioning was accomplished with a Spencer microtome adapted for thin sectioning by the method of Pease & Baker (1948). Razor blades were sharpened to an angle of 52° and used for the cutting edge. The sections were cut with the microtome set for one-twentieth of a micron and then placed directly on electron microscope grids and observed in a model 50 R.C.A. electron microscope.

RESULTS

Plates 1 and 2, and Pl. 3, figs. 3 and 4, show osmic acid-fixed and sectioned cells of *Saccharomyces carlsbergensis*. In all cases where the protoplasm shrank away from the cell wall, at least two lines are shown in the cell-wall area. In the areas where the protoplasm remained in contact with the cell wall, the inner line was difficult to detect, but could be seen in some pictures. The usual picture obtained by sectioning is represented by Pl. 2, fig. 2. The two areas seen in the cell wall are consistent with the double membrane cell-wall structure demonstrated for *S. cerevisiae* by Northcote & Horne (1952). If the limits of the two cell-wall membranes shown in Pl. 2, fig. 2 are taken, the cell-wall width measures 0.16 μ . Northcote & Horne believed that possibly more than two membranes were present, since they reported seeing several thin layers peeling away at the site of the bud scars following the treatment of their cell-wall preparations with HCl. A similar peeling is indicated in our pictures by the arrows at the site of the new bud shown in Pl. 3, fig. 6, which is just beginning to push through the old cell wall of the mother cell. Additional evidence for several layers in the cell wall is indicated by the laminations in the cell wall indicated by the arrows in Pl. 1, fig. 1 and Pl. 3, fig. 3. The outer and inner membranes of the cell wall seem to consist of closely packed thin layers of material, whereas the less dense central area seems to consist of less well-developed layers, not so closely packed together. The bottom part of the cell shown in Pl. 3, fig. 4 (see arrow) is interesting since it shows a cut directly through a bud scar. Note that the double cell-wall membrane persists through the bud scar.

A comparison of the cell-wall development shown by the mother and daughter cells in Pl. 3, fig. 6, is of interest. Henrici (1947) stated that the cell wall of young cells is thin and increases in thickness with age. This is clearly indicated in Pl. 3, fig. 6, since the mother cell has a rather well-

developed cell wall, while the cell wall of the daughter cell is comparatively thin. This then is direct proof that the cell wall does increase in thickness with age, and the thick cell wall shown in Pl. 4, fig. 7 represents the cell wall of an older cell (or 'dauerzellen' of the older literature).

Plate 3, figs. 5 and 6, are sections through osmic acid-fixed cells of *Saccharomyces cerevisiae*. Note the presence of the inner cell-wall membrane shown in Pl. 3, fig. 5. The double cell-wall membranes are faintly shown in Pl. 3, fig. 6, but *S. cerevisiae* did not give as clear results as did *S. carlsbergensis*. Pl. 4, figs. 8–10, show sections of *S. carlsbergensis* in which osmic acid fixation was omitted. Notice that the cell wall is not nearly as well delineated as in the sections where osmic acid was used. In fact, little of the double membrane nature of the cell wall is in evidence. It is also obvious that the protoplasm was not nearly as shrunken nor as opaque to the electron beam as when osmic acid was used as a fixative. This resulted in the demonstration of some interesting electron-opaque bodies in the cell vacuole. They appeared either large, single, or paired, as in Pl. 4, figs. 9 and 10; or small paired, or in chains, as in Pl. 4, fig. 8. Attempts were made to identify these bodies as volutin by extraction from the intact cell by appropriate methods before embedding and sectioning, but these procedures resulted in cells which were valueless for observation after sectioning. These bodies are identical in appearance with those termed mitochondria by Sarachek & Townsend (1953), but undoubtedly might also be the structures often referred to as volutin, lipoidal granules, metachromatic granules, or even possibly the chromosomes of Subramanian & Ranganathan (1945).

The sectioned cells shown here are all from 72 hr. cultures; when 24 hr. cultures were used the resulting preparations gave such poor quality sections that little could be determined in regard to cell structures.

DISCUSSION

The pictures shown in this paper appear to confirm earlier reports of the presence of at least two membranes in the yeast cell wall. However, it must be admitted that some problems of interpretation of the pictures exist. Some could argue that the outer line shown in the photographs represents adsorbed dirt, and the inner line represents adhering protoplasm. We do not believe this to be the case since these lines did not appear in the cell walls of similarly treated cells not fixed with osmic acid (compare Pl. 2, fig. 2 and Pl. 4, fig. 8), and all of the cells were washed several times before the embedding steps were begun. In addition, Guilliermond (1920) reported Will's statement that the double membranes in the cell wall were made more visible by treatment with osmic acid. One would expect that this would hold also for electron microscopy since osmic acid is known to make materials electron-opaque. Thus the osmic acid could act as a stain for these membranes. Close inspection of the enlarged cell wall shown in Pl. 3, figs. 3 and 5 (and to a lesser extent in Pl. 1, fig. 1), shows wavy structures constituting the inner membrane which would be difficult to explain on the basis of adhering protoplasm.

It seems highly probable that these pictures give cytological confirmation of the two separate permeability barriers reported for the yeast cell wall by Conway & Downey (1950) who used physical chemical methods. If one calculates the cell volume included by the two cell-wall membranes shown in Pl. 1, fig. 1, it turns out to be *c.* 10 % of the total cell volume. This is in agreement with Conway & Downey who found by their calculations that the yeast cell wall was bounded by two different permeability barriers and that the cell wall occupied from 10 to 11 % of the total cell volume. It logically could be concluded that the outer line shown in our pictures represents the outer permeability barrier (impermeable by inulin and peptone, but permeable by succinic, glyceric, acetic and propionic acids), and the inner line in our pictures represents the inner permeability barrier (impermeable by succinic and glyceric acids, but permeable by acetic and propionic acids) reported by Conway & Downey.

The question can arise about the possibility of the inner line representing the cytoplasmic membrane of the yeast cell. Robinow & Murray (1953) demonstrated the cytoplasmic membrane of bacteria and yeast by a staining method and observation with the light microscope. In their pictures the cytoplasmic membrane adhered to the protoplasm and shrank away from the cell wall on plasmolysis. It is obvious that the inner cell-wall membrane demonstrated by our pictures is sufficiently rigid to remain as an outer structure after the protoplasm has retracted. For this reason we believe it to be part of the cell wall and not the cytoplasmic membrane. It is not surprising that a cytoplasmic membrane was not demonstrated in our sections of yeast since Chapman & Hillier (1953) also failed to demonstrate it in their sections of bacteria. It is possible either that the membrane was too small for resolution, that it lacked electron-scattering powers, or that it was destroyed by the treatments involved in embedding and sectioning.

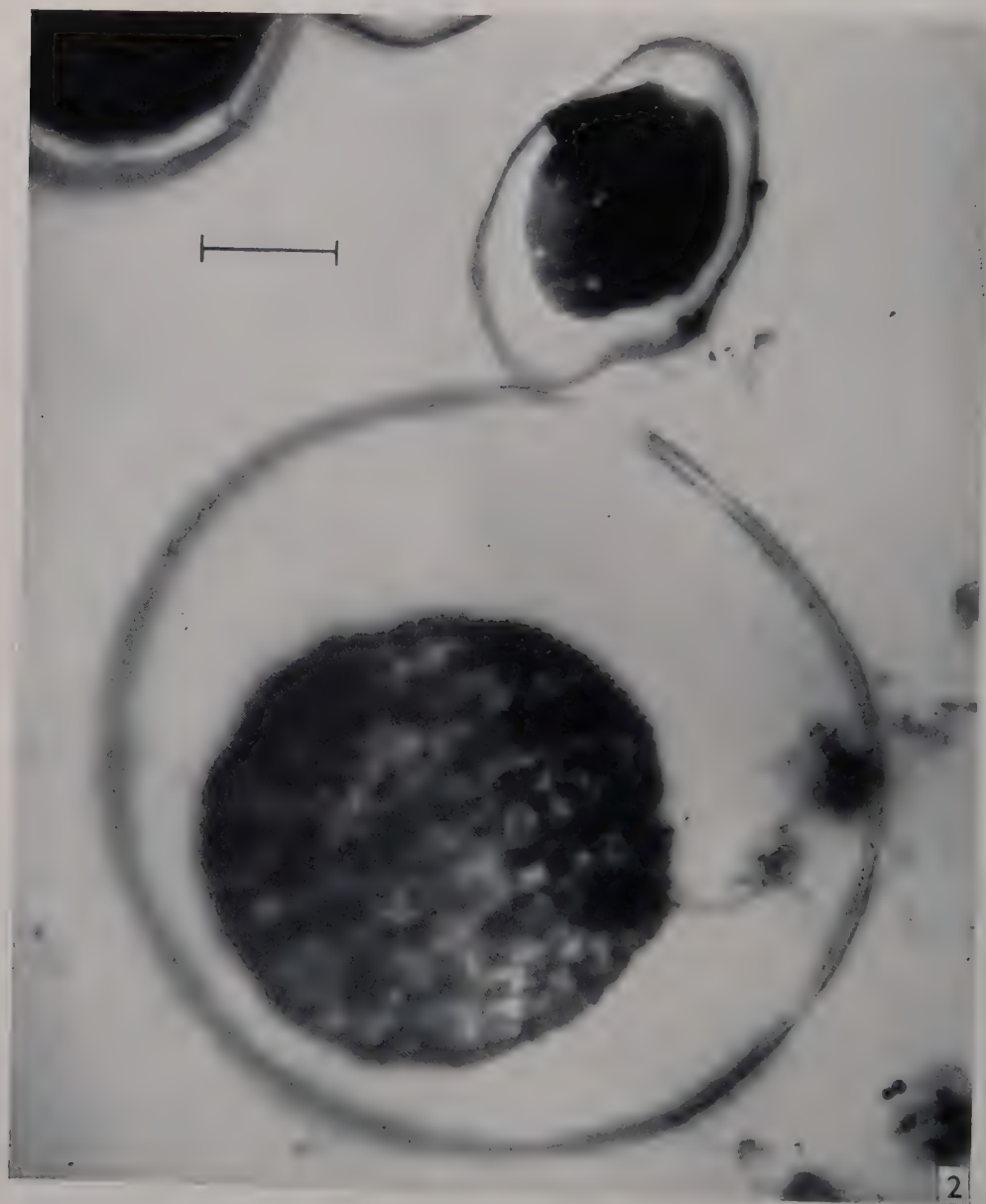
The electron opaque structures shown in Pl. 4, figs. 8-10, are interesting. These have the same appearance as the 'mitochondria' reported for yeast cells by Sarachek & Townsend (1953). These authors reported that the 'mitochondria' were small in actively metabolizing yeast, and that they coalesced into larger particles as metabolism slowed. This would mean that the smallness of the particles in Pl. 4, fig. 8, would be due to a high metabolic rate for this cell at the time of fixation, while the larger particles shown in Pl. 4, figs. 9 and 10 would be due to their presence in an older cell with a lower metabolic rate. Sarachek & Townsend reported that the large forms originated by coalescence of the smaller bodies, and they would interpret the paired bodies shown in Pl. 4, fig. 10, as being in the act of coalescence. However, it is not certain that these structures should be called mitochondria, and it would be just as well to call them volutin, lipoidal granules, or metachromatic granules. It is also possible that these are the tennis-ball-shaped chromosomes of some authors (Subramanian & Ranganathan, 1945). We have not yet been able to identify the nature of these particles, but we can report that they are highly volatile under an intense electron beam since they often disappeared as they were being observed in the electron microscope.



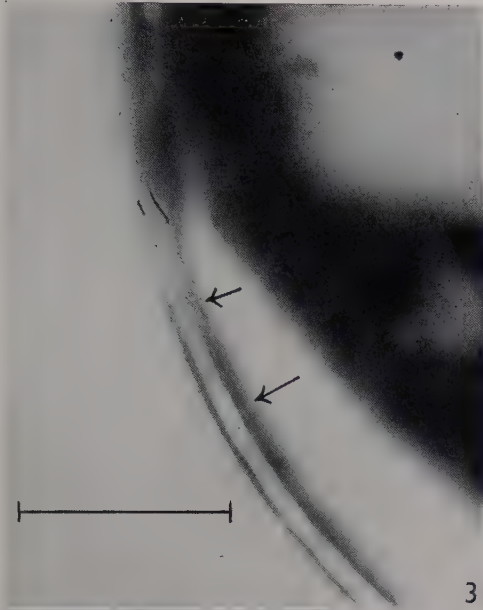
On all figures the length of the scale is 1μ .

J. W. BARTHOLOMEW & R. LEVIN—YEAST STRUCTURE AS SHOWN BY ULTRA-THIN SECTIONING.
PLATE I

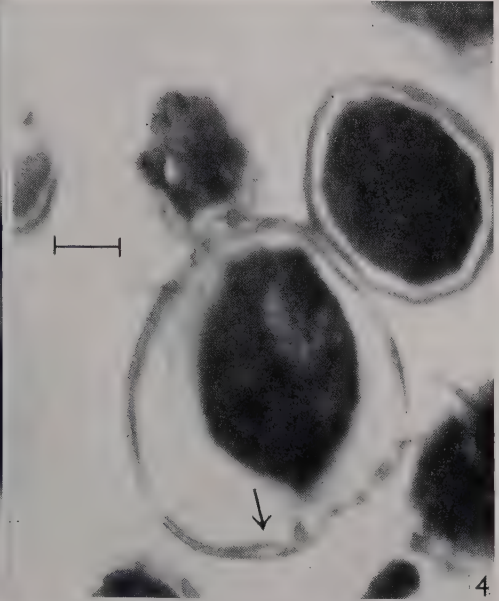
(Facing p. 476)



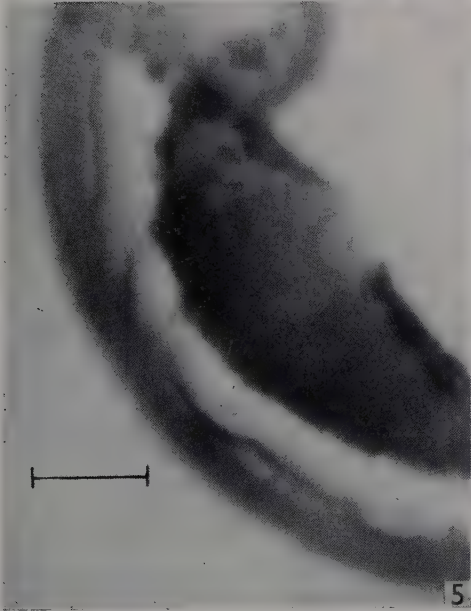
J. W. BARTHOLOMEW & R. LEVIN—YEAST STRUCTURE AS SHOWN BY ULTRA-THIN SECTIONING.
PLATE 2



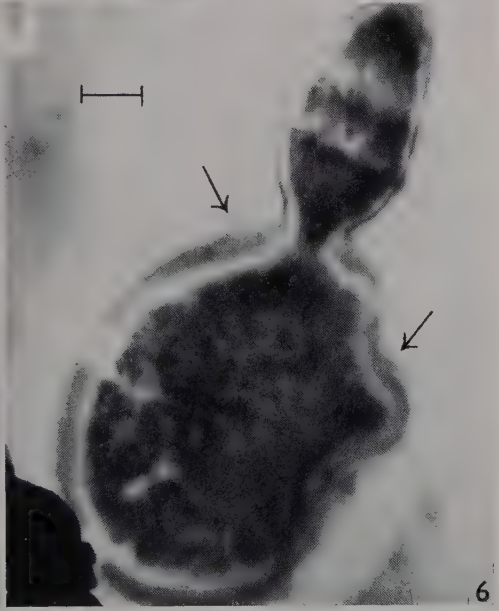
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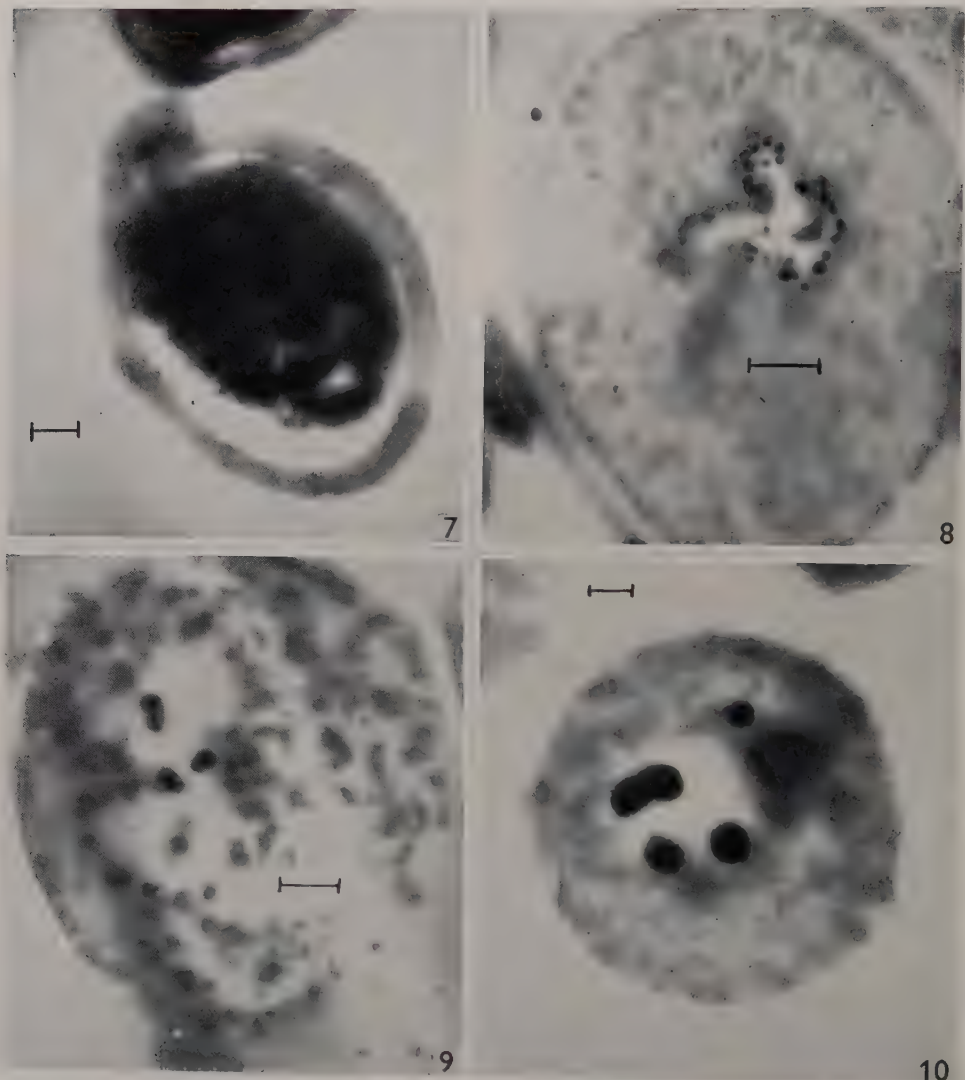


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J. W. BARTHOLOMEW & R. LEVIN—YEAST STRUCTURE AS SHOWN BY ULTRA-THIN SECTIONING.
PLATE 3



J. W. BARTHOLOMEW & R. LEVIN—YEAST STRUCTURE AS SHOWN BY ULTRA-THIN SECTIONING.
PLATE 4

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. *S. carlsbergensis*, osmic acid fixed, embedded in plastic, sectioned at one-twentieth of a micron. Showing the double membrane nature of the cell wall.

PLATE 2

Fig. 2. Same as fig. 1.

PLATE 3

Fig. 3. An enlarged portion of fig. 2.

Fig. 4. *S. carlsbergensis*, same treatment as figs. 1 and 2. Showing a section through a bud scar, and the double membrane nature of the cell wall.

Fig. 5. *S. cerevisiae*, osmic acid fixed, embedded in plastic, sectioned at one-twentieth of a micron. Showing cell-wall structure. Note the fibrous nature of the inner cell-wall membrane.

Fig. 6. Same as fig. 5. Showing a section through a young bud, and a section through two newly forming buds.

PLATE 4

Fig. 7. *S. carlsbergensis*, same as figs. 1 and 2. Showing the thick cell wall of an old cell.

Figs. 8-10. *S. carlsbergensis*, osmic acid-fixation step omitted, otherwise the same as fig. 1. Showing internal electron-opaque granules which were masked when osmic acid was used as a fixative. These granules have been variously termed volutin, mitochondria, chromosomes and lipoidal granules.

The length of the scale in each figure is 1 μ .

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The Decomposition of 1-Chloro- and 1-Bromonaphthalene by Soil Bacteria

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SUMMARY: Two species of naphthalene-utilizing bacteria from soil were able to metabolize 1-chloro- and 1-bromonaphthalene. From cultures in which 1-chloronaphthalene was the sole source of organic carbon, D-8-chloro-1:2-dihydro-1:2-dihydroxynaphthalene and 3-chlorosalicylic acid were isolated. 3-Bromosalicylic acid was isolated from cultures with 1-bromonaphthalene and evidence of the formation of a 'diol' compound obtained. The course of the metabolic pathways is discussed.

In previous work, Treccani, Walker & Wiltshire (1954) studied 5 strains of bacteria, obtained from soil, which could grow with naphthalene as their only supply of organic carbon. Evidence was presented that D-*trans*-1:2-dihydro-1:2-dihydroxynaphthalene, salicylic acid and catechol were successive metabolic intermediates. These organisms also grow in a mineral salts medium with 1-chloronaphthalene as the carbon source; the study of the metabolism of this compound and of 1-bromonaphthalene forms the subject of the present paper. The two organisms which we used in cultures for the isolation of metabolic intermediates are referred to as organism I and organism II. Some of these results have already been reported briefly (Walker & Wiltshire, 1953*b*).

METHODS

Bacteria. Organism I was described by Walker & Wiltshire (1953*a*). Organism II was considered by Treccani *et al.* (1954) to be a strain of *Pseudomonas desmolyticum*, Gray & Thornton (1928).

Media. The mineral salts medium used by Tausson (1927), but adjusted to pH 6.9, was used, with addition of 1-chloronaphthalene or 1-bromonaphthalene (both British Drug Houses Ltd. laboratory grade reagents).

Culture conditions. Stock cultures were maintained on plates of Tausson's medium solidified with agar (2%, w/v) and inverted over naphthalene crystals. Cultures in liquid media were grown in 1 l. Roux bottles containing 100 ml. salts solution and one or two drops of 1-chloro- or 1-bromo-naphthalene. Incubation was usually at 25°. Some cultures were grown at room temperature in 6 l. round-bottomed flasks containing 4 l. medium stirred mechanically.

Detection of intermediates. The reactions for the detection of chloro- and bromo-naphthalene diols and of chloro- or bromo-salicylic acids were those used by Walker & Wiltshire (1953*a*) for the corresponding unsubstituted compounds.

Absorption spectra. The absorption spectra of several chloro-naphthols were measured in the range 240-360 m μ ., using a Unicam S.P. 500 spectrophotometer.

Reference compounds

4-Chloro-1-naphthol was prepared by the method of Kast (1911).

5-Chloro-1-naphthol was synthesized by the method of Erdmann & Kirchhoff (1888). It was also prepared from 5-nitro-1-chloronaphthalene (Hodgson & Walker, 1933) by reduction to 5-chloro-1-naphthylamine followed by diazotization and hydrolysis of the diazonium group.

8-Chloro-1-naphthol was synthesized from 8-nitro-1-naphthylamine (prepared by the method of Hodgson & Davey, 1939), by replacement of the amino group by chlorine using the procedure of Hodgson & Walker (1933), followed by reduction to 8-chloro-1-naphthylamine, diazotization and hydrolysis of the diazonium group. (8-Chloro-1-naphthol could not be obtained by the hydrolysis of 8-nitro-1-chloronaphthalene with boiling water as reported by Woroshtzow & Koslow (1936) nor when water was replaced by dilute aqueous sulphuric acid containing a little urea or by dilute aqueous sodium carbonate.)

8-Chloro-2-naphthol was obtained by replacing the amino group of 7-methoxy-1-naphthylamine by chlorine in the usual Sandmeyer procedure and demethylation of the resulting 7-methoxy-1-chloronaphthalene using 48% aqueous hydrobromic acid (Pope & Woodcock, unpublished; cf. James & Woodcock, 1951).

3-Chlorosalicylic acid was synthesized in very small yield by a Kolbe reaction on *o*-chlorophenol.

3-Chloro-2-acetoxybenzoic acid. 3-Chlorosalicylic acid (70 mg.) was heated for 5 min. with a few drops of acetic anhydride and, after cooling, water was added, the mixture heated until clear and then filtered. On standing, the filtrate deposited glistening colourless plates, m.p. 122–123° (18 mg.). (Found: C, 50.22; H, 3.08%; $C_9H_7O_4Cl$ requires: C, 50.35; H, 3.26%.)

RESULTS

Isolation and constitution of the chloronaphthalene diol

When the diol concentration in Roux bottle cultures of organism I growing on chloronaphthalene was at a maximum (usually after 4–6 days of incubation) the culture fluid was collected, neutralized with aqueous sodium hydrogen carbonate, heated to about 60° to flocculate the inorganic phosphate precipitate and cells, and filtered; residual chloronaphthalene remained on the filter. To each litre of clear yellow filtrate, 1 g. purified animal charcoal (British Drug Houses Ltd.) was added, and after thorough shaking, the charcoal was filtered off and dried at room temperature; the filtrate was retained for isolation of chlorosalicylic acid (see below). The dried charcoal was extracted for 8 hr. in a Soxhlet apparatus with a mixture (1:1) of ethanol and benzene, and the extract evaporated to dryness under reduced pressure. The residue was dissolved in water, filtered and the solution extracted repeatedly with ether. The combined ethereal extracts were dried (Na_2SO_4), filtered and concentrated. From the concentrated solution, chloronaphthalene

diol crystallized out and was recrystallized from benzene in colourless needles, m.p. 108° , $(\alpha)_{\text{D}}^{20} + 77^{\circ}$, $c = 0.9$ in EtOH. (Found: C, 60.54; H, 4.6; Cl, 18.17%; $\text{C}_{10}\text{H}_9\text{O}_2\text{Cl}$ requires: C, 61.06; H, 4.6; Cl, 18.07%. E_{max} , 8.9×10^{-3} at $263 \text{ m}\mu$. in 95% ethanol, 2.0×10^{-3} at $293 \text{ m}\mu$. and 1.8×10^{-3} at $305 \text{ m}\mu$.)

Constitution of the chloronaphthalene diol. The chloronaphthalene diol behaved like naphthalene diol in being dehydrated to a phenol by boiling for a few minutes with N-HCl. When the resulting solution was adjusted to pH 10 and treated with *n*-butanol and 2:6-dichloroquinonechloroimide (phenol reagent) a blue colour becoming greener on standing was produced in the butanol layer, but no colour was given without previous acid treatment. The rate of colour development was slightly slower than with 1-naphthol which gives a blue colour. 8-Chloro-1-naphthol behaved like 1-naphthol, but 8-chloro-2-naphthol gave a slowly developing green colour.

The presence of two phenolic compounds after acid treatment of the chloronaphthalene diol was demonstrated by running paper chromatograms, using 0.1N aqueous ammonia as the developing solvent (Boyland & Sims, 1953). The spots were detected by spraying the paper after drying with a solution of diazotized *p*-nitroaniline followed when dry with aqueous sodium carbonate. As controls, drops of 2% ethanolic solutions of 8-chloro-1-naphthol, 8-chloro-2-naphthol and an equal mixture of the two were used. The R_f value of 8-chloro-1-naphthol was slightly greater than that of 8-chloro-2-naphthol. These observations suggested that acid treatment of the chloronaphthalene diol gave a mixture of mainly 8-chloro-2-naphthol with some 8-chloro-1-naphthol.

A similar experiment with *d*-trans-1:2-dihydro-1:2-dihydroxynaphthalene from a naphthalene culture, and from the urine of a rabbit dosed with naphthalene and with solutions of 1- and 2-naphthols as controls, showed that the naphthalene diol gave a mixture of 1-naphthol with a very small proportion of 2-naphthol on acid treatment.

Dehydration of chloronaphthalene diol. The diol (21 mg.) was treated with 4N-HCl on a boiling water bath for 5 min. and the solution concentrated to dryness. The residue (19.6 mg.) was taken up in dilute aqueous sodium hydroxide, the solution filtered and acidified with HCl. The white precipitate that formed was collected, dried and sublimed under reduced pressure at 100° , affording colourless needles, m.p. $97-98^{\circ}$. On admixture with authentic 8-chloro-2-naphthol (m.p. 100°) the m.p. was $98-99^{\circ}$. The m.p. was depressed on admixture with 8-chloro-1-naphthol. In another experiment, a yield of 3 mg. 8-chloro-2-naphthol (37% theory) was obtained from 9 mg. chloronaphthalene diol by the above procedure.

Supporting evidence of the identity of the chloronaphthol isolated above was obtained by determining its absorption spectrum in the ultraviolet region (240–360 $\text{m}\mu$). Three distinct zones of absorption, which is a characteristic of 2-naphthols (cf. Daglish, 1950) were distinguished. Comparison with the spectrum of authentic 8-chloro-2-naphthol showed that the spectra were similar, although this evidence is not decisive since 1-chloro-2-naphthol, for example, also has a similar spectrum. The latter compound has a different m.p., 70° (Cleve, 1888). No other monochloro-2-naphthols were available.

The spectrum of the reaction mixture from acid-treated chloronaphthalene diol was compared with that of mixtures of 8-chloro-1- and 8-chloro-2-naphthols; it appeared that the reaction mixture contained about 10 % of 8-chloro-1-naphthol. Since the chloronaphthalene diol on treatment with dilute acid yielded 8-chloro-2-naphthol and very probably 8-chloro-1-naphthol, it is concluded that the diol is a *dextro* form of 8-chloro-1:2-dihydro-1:2-dihydroxy-naphthalene.

Isolation of 3-Chlorosalicylic acid

The filtrate from the charcoal used for adsorbing the chloronaphthalene diol was acidified with dilute HCl and treated with 1 g. animal charcoal/l. After filtering off, the charcoal was dried and eluted with boiling ethanol or dilute aqueous sodium hydrogen carbonate. In the former case, the ethanol was evaporated and the residue dissolved in aqueous sodium hydrogen carbonate. This solution was then extracted with ether to remove non-acidic material, and the aqueous layer separated, acidified with HCl and again extracted with ether. This ethereal extract was dried (Na_2SO_4), filtered and the solvent removed giving crystalline 3-chlorosalicylic acid. This was purified by vacuum sublimation at 120° and recrystallization from water, affording colourless needles, m.p. 178° , not depressed on admixture with an authentic specimen. (Found: Cl, 20.3 %; calc. for $\text{C}_7\text{H}_5\text{O}_3\text{Cl}$, Cl, 20.58 %.) Addition of aqueous FeCl_3 to a dilute solution of the acid gave an intense violet colour. On treatment of the acid with hot acetic anhydride, followed by crystallization from water, a colourless crystalline acetyl compound, m.p. $122\text{--}123^\circ$, not depressed on admixture with authentic 3-chloro-2-acetoxybenzoic acid, was obtained.

Bromonaphthalene diol

After about 2 or 3 days incubation at 25° cultures of organism I or II growing on 1-bromonaphthalene gave a weak blue phenol reaction after boiling for 2 min. with N-HCl. The compound responsible for this test was extracted from neutral aqueous solution by ether, and although it has not been isolated or examined further, it is probably a bromonaphthalene diol.

Isolation of 3-bromosalicylic acid

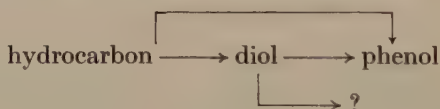
Eight Roux bottles containing 125 ml. Tausson's medium and 2 drops of 1-bromonaphthalene were inoculated with organism I and incubated at 25° . After 5 days all the cultures were yellow and gave a weak violet ferric reaction. The cultures were collected, concentrated under reduced pressure to about a tenth of their volume, and after acidifying with dilute HCl, were extracted 3 times with ether. The combined ethereal extracts were washed with a little water, then extracted with dilute aqueous sodium hydrogen carbonate. The aqueous layer was separated, acidified with dilute HCl and extracted with ether. The ether layer was separated, dried (Na_2SO_4) and the ether removed leaving a brownish solid (7 mg.) which was crystallized from water (charcoal) giving nearly colourless crystals (4 mg.), m.p. $130\text{--}150^\circ$. The material obtained

from three such batches of cultures was combined, sublimed *in vacuo* at 140° , and the sublimate was crystallized from water in colourless needles, m.p. 181° . (Found: C, 39.41; H, 2.375; Br, 36.8% calc. for $C_7H_5O_3Br$: C, 38.71; H, 2.305; Br, 36.87%. Müller (1909), found m.p. 184° for 3-bromosalicylic acid.) Addition of $FeCl_3$ to a dilute solution of the acid produced a deep violet colour.

DISCUSSION

Of the three possible 1-chloronaphthalene diols, the product isolated from chloronaphthalene cultures was 8-chloro-1:2-dihydro-1:2-dihydroxynaphthalene, the orientation of which was established by dehydration in dilute acid solution to chloronaphthol. In contrast to the formation of 1-naphthol from unsubstituted 1:2-dihydro-1:2-dihydroxynaphthalene (Young, 1947), the chlorinated diol yielded mainly a 2-naphthol. The formation of a small amount of 8-chloro-1-naphthol was shown by paper chromatography and explains the initial appearance of a blue colour, later becoming greenish blue, in the phenol reaction with acid-treated chloronaphthalene diol.

Treccani *et al.* (1954) found that the metabolism of naphthalene by five different strains belonging to at least three species of soil bacteria followed a similar course. All the strains can also grow on 1-chloronaphthalene, producing a diol and a chlorosalicylic acid, but no chloronaphthol. Diol formation appears to be a general reaction in the oxidation of polycyclic aromatic hydrocarbons, but little is known about the subsequent metabolism of such diols. Their ready dehydration to phenols, and the isolation of phenol conjugates from the urine of mammals dosed with hydrocarbons has led to the belief that both phenols and diols are metabolites, e.g.



With bacteria there is no evidence that naphthols are formed from naphthalene, and the recent isolation of naphthalene diol glucuronide (Corner, Billett & Young, 1954) which accounted for nearly all the 'extra glucuronide' in naphthalene-dosed rabbit urine suggests that what had previously been estimated as 1-naphthol glucuronide from animals may well have been diol glucuronide. The evidence from both bacteria and animals favours the conclusion that the diols are true metabolic intermediates, whereas naphthols may be artefacts or at best arise from side-reactions. The intermediate compounds between naphthalene diol and salicylic acid remain to be identified. On this point, the study of the metabolism of 1-chloronaphthalene indicates that: (1) oxidation occurs in the ring not substituted with chlorine, since 8-chloro-1:2-dihydro-1:2-dihydroxynaphthalene is the diol formed; (2) the carbocyclic ring so de-aromatized is broken first, giving rise to a chlorosalicylic acid; (3) carbon atom 4 in 8-chloro-1:2-dihydro-1:2-dihydroxynaphthalene becomes the carboxyl carbon atom of 3-chlorosalicylic acid. It is still not known at which bond opening of the ring occurs.

Although growth of organism I or II in 1-bromonaphthalene media is slower than with 1-chloronaphthalene, the metabolic pathway is similar, since a diol compound can be detected and 3-bromosalicylic acid accumulates in the cultures. The bromonaphthalene diol has not been isolated, but it seems reasonable to expect this to be 8-bromo-1:2-dihydro-1:2-dihydroxynaphthalene.

We are grateful to Dr D. Woodcock for a gift of 8-chloro-2-naphthol, and to Dr H. G. Thornton, F.R.S., for his interest and encouragement.

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The Influence of Bacteriophage on *Streptococcus pyogenes*

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SUMMARY: By the selective action of certain bacteriophages on cultures of susceptible streptococci it is possible to alter or enhance certain of their characteristics. The extractable M antigen may be greatly increased; non-mucoid strains may become completely mucoid with or without the increase in M antigen. Some phage-selected cultures have shown a marked increase in mouse virulence and some an ability to survive in normal human blood not possessed by the parent strain.

Despite the great amount of work published on bacteriophages, the only extensive studies on those active against group A haemolytic streptococci are those by Evans (e.g. 1933, 1934, 1940) and Evans & Sockrider (1942). In preliminary work with phages obtained from sewage and sent to us by Professor Boulgakov of Paris, it was found that lysis was often much more readily obtained when the test was carried out in the presence of hyaluronidase (Maxted, 1952). It was also noted that the colonies of the secondary growth that appeared after vigorous lysis had taken place were often very mucoid when plated on to glucose serum agar, even when the parent strain had never shown mucoid variants upon preliminary plating. The secondary growth from one strain also showed increased M antigen content, and an ability to survive in human blood not possessed by the parent strain. A more detailed investigation was therefore made of the changes in antigenic structure, colonial appearance and virulence that are produced in cultures by passage through phage-containing media.

MATERIAL AND METHODS

Preparation of hyaluronidase. Minced bull testicle was extracted overnight in the cold with N/1 acetic acid, neutralized, and the supernatant fluid Seitz-filtered.

Source and propagation of phages. The phages used were derived from those isolated by Professor Boulgakov from Paris sewage; they were propagated in nutrient broth containing hyaluronidase, each on its particular strain of streptococcus. The phages are designated A1, A6, A12 and A25. The letter indicates the Lancefield group to which the propagating strain belongs and the number the type. The phages are not specific for their propagating type strain.

For the phage passage experiments with types 3 and 27 the phage A1 was adapted to these strains; for the experiments with the type 5 strain phage A6 was similarly adapted. A mixture of all four of the original phages was used for the serial transfers of strain 1506 type 30.

Serial phage passage. Two separate 5 ml. amounts of nutrient broth, containing 3 % of crude hyaluronidase extract, were inoculated with a loopful of 18 hr. Todd-Hewitt broth culture. To one tube, 0.02 ml. of the appropriate phage filtrate was added, the other acted as a control and both tubes were then incubated overnight. Each was subcultured into fresh hyaluronidase broth and on to serum glucose agar. This procedure was repeated as often as necessary.

Phage survivor. The term phage survivor was applied to those strains which had survived passage through broth containing both phage and hyaluronidase, in tests in which strong and visible lysis had taken place. When transferred to Todd-Hewitt broth in the absence of hyaluronidase, they grew vigorously. They were not necessarily resistant to phage when grown under conditions most favourable to phage action.

Detection of mucoid colonies. The presence of mucoid colonies was determined in 15–18 hr. cultures on nutrient agar containing 0.5 % glucose and 5 % horse serum. On such a medium many streptococci form somewhat mucoid colonies, but except for certain serological types (e.g. 17, 18, 19 and 24) they do not ordinarily produce the 'grossly mucoid' colonies characteristic of many of the strains that survive phage passage.

Estimation of hyaluronic acid. Samples of broth supernatant from cultures grown in Hartley digest broth with 3 % maltose added were tested every 24 hr. over a 4-day period. Estimations of hyaluronic acid were by the acidified serum turbidimetric method (Faber & Rosendal, 1954). Commercial potassium hyaluronate was used for the standard hyaluronic acid solution.

Detection of type antigens. Cultures grown in 50 ml. amounts of buffered glucose broth were centrifuged and the deposit extracted with hot acid, neutralized, and tested in capillary tubes against specific antisera (Swift, Wilson & Lancefield, 1943). Agglutination tests for T antigen were carried out on suspensions grown at 30° in a buffered glucose broth, and digested with pancreatic extract to render them smooth and to destroy the M antigen.

The antisera for both the precipitation and agglutination tests were those used for routine type identification of streptococci, and prepared by the recognized techniques of Swift *et al.* (1943) and Griffith (1934).

Estimation of mouse virulence. Tenfold dilutions of overnight cultures, grown in a buffered glucose broth, were inoculated intraperitoneally, in 0.5 ml. amounts, into mice which were subsequently observed for 10 days.

Bactericidal tests. Tubes ($2 \times \frac{1}{2}$ in.) containing 0.3 ml. of normal heparinized human blood were inoculated with 0.02 ml. of suitable dilutions (usually 10^{-3} and 10^{-5}) of the culture under test. With the higher dilution the inoculum usually contained between 30 and 100 viable units. The tubes were corked and incubated at 37°, being shaken mechanically for 3 min. every 15 min. Explants were made in duplicate at 3 and 20 hr. over one-sixth of a 10 cm. diam. blood agar plate, using a standard loop throughout. The growth was recorded as \pm to + + + +.

Source of strains. Strain T5B (type 5), S43 (type 6) and SF40 (type 27) were from the original Griffith collection. Strain Richards (type 3) came from a case of puerperal fever; strains J.L., 927, 1518, 1608 and 1130 are all recently

isolated strains of type 12; J.L. and 1518 were isolated from the throats of patients with acute nephritis, 1608 and 1130 from cases of tonsillitis and 927 from the throat of a symptomless carrier. Strain 1506 (type 30) was isolated from the throat of a child in a rheumatic fever convalescent home.

RESULTS

Colonial change after phage passage

With some strains of *Streptococcus pyogenes* the phages produced no change in colonial appearance, but, provided their lytic action was strong, many strains showed a profound change to a grossly mucoid colony form (Pl. 1, fig. 1). The investigations reported here have been concerned very largely with the strains which showed this change.

Of 50 non-mucoid strains of streptococci examined for phage survivors, 36 yielded mucoid variants. These 36 strains were distributed over 13 different types.

Strains which normally tend to yield mixtures of mucoid and non-mucoid colonies usually yielded none but mucoid colonies after phage passage. The latter were often larger and remained raised and moist on a solid medium longer than mucoid colonies selected visually from the mixed parent strain. The change after phage passage is most striking with strains that ordinarily show no mucoid colonies on culture, such as the strain 1130 (type 12) which has been used in many of the experiments described.

Table 1. *Hyaluronic acid in the supernatant fluid of cultures of phage-passaged strains and their non-mucoid parent strains*

Strain	Type		Hyaluronic acid, mg./ml., after incubation for			
			24 hr.	48 hr.	72 hr.	96 hr.
T5 B	5	Parent	0	0	0	0
		Phage survivor	0.3	0.26	0.16	0.02
S43	6	Parent	0	0	0	0
		Phage survivor	0.27	0.27	0.31	0.3
SF40	27	Parent	0	0	0	0
		Phage survivor	0.21	0.15	0.04	0
1130 matt	12	Parent	0	0	0	0
		Phage survivor	0.35	0.35	0.35	0.35
1130 glossy		Parent	0	0	0	0
		Phage survivor	0.38	0.38	0.41	0.35

The mucoid appearance of the colonies is probably due to increased production of the hyaluronic acid which ordinarily forms the streptococcal capsule. Thus colonies of phage-surviving mucoid variants, as well as naturally occurring mucoid variants, had non-mucoid colonies on agar containing hyaluronidase (Pl. 1, fig. 2). With all of five strains tested, the phage-survivors liberated hyaluronic acid into the medium during growth, although none was detected in cultures of their non-mucoid parent strains (Table 1). The progressive decrease in hyaluronic acid in the T5B and T27 phage-survivor

cultures was due to weak hyaluronidase production; none of the other strains produced detectable amounts of the enzyme.

To discover the rapidity with which the mucoid variants appeared, tubes of nutrient broth with and without phage were seeded with strain 1180 (type 12). Counts were made at intervals by plating on serum glucose agar to which phage antiserum had been added. Phage-particle counts were also made on plates previously flooded with the propagating strain for this phage. The first mucoid colonies appeared between 2 and 4 hr. (Table 2) and at 6 hr. the organisms present in the phage broth, though only slightly more numerous than the original inoculum, were all mucoid. The change occurred during the period of greatest phage multiplication.

Table 2. *Time at which mucoid variants appeared in broth culture with phage present*

Time	Numbers of colonies/ml.					No. phage particles/ ml $\times 10^4$
	Control tube, no phage		Broth + phage			
	Non-mucoid $\times 10^4$	Mucoid	Total $\times 10^4$	Non-mucoid (%)	Mucoid (%)	
15 min.	0.7	0	0.7	100	0	5
2 hr.	6	0	5.5	100	0	9
4 hr.	58	0	0.2	84	16	150
6½ hr.	320	0	1.1	0	100	310
9 hr.	240	0	15	0	100	300

Origin of mucoid variants

Most of the non-mucoid strains which developed mucoid colony variants after phage passage were also made mucoid by passage through mice. This, and the fact that many strains of streptococci are found normally to yield a mixture of mucoid and non-mucoid colonies, suggested that the appearance of completely mucoid cultures after phage passage was probably due to selection.

In an attempt to distinguish between selection by the phages of randomly appearing mutants and the production of variants by the action of the phage, experiments were made on the lines suggested by Newcombe (1949) and by Luria & Delbrück (1943).

In the first, three to nine 15 cm. plates of glucose serum agar were inoculated with approximately 1500 viable units of the streptococcus under test and incubated at 37° for 3–4 hr. The cells on half the plates were then re-distributed by spreading 0.5 ml. of saline over the surface with a glass spreader; the other plates remained as controls. Both sets were carefully flooded with a phage filtrate known to be lytic for the streptococcus. (In other experiments it was shown that the flooding did not lead to appreciable spreading of the colonies.) After incubation the numbers of mucoid colonies on both sets of plates were counted (Table 3). In two of the four tests there were clearly more mucoid colonies on the 'spread' plates than on the 'unspread' plates, as would be expected on the supposition of a random-mutant origin for the mucoid

variants. In the other two experiments, however, there was no noteworthy difference between the spread and unspread plates. The test could not therefore be held to give convincing evidence on the origin of the variants.

Table 3. *Number of mucoid colonies developing on spread and unspread phage-impregnated plates, in four spread-plate experiments*

Strain	Type		Colonies on control plate, without phage	No. of mucoid colonies on individual phage-containing plates										Mean no.
T5 B	5	Control	1600	1	1	1	0	4	2	1	3	2	1.7	
		Spread	Confluent	5	6	6	1	4	7	6	1	11	5.2	
SF40	27	Control	2000	5	3	4	3	4	2	6	5	.	4.0	
		Spread	Confluent	0	6	3	5	2	6	7	0	.	3.6	
1 matt	12	Control	1500	10	9	14	6	11	10.0	
		Spread	Confluent	5	12	16	7	6	9.2	
2 matt	12	Control	1200	0	0	1	0.3	
		Spread	Confluent	7	11	6	8.0	

Two strains were submitted to Luria & Delbrück's fluctuation tests. Twelve 5 ml. broth tubes were inoculated with 0.02 ml. samples of the culture previously diluted 10^{-3} , and incubated for 3–4½ hr. Single 0.4 ml. samples from each tube were then transferred to glucose serum agar plates containing sufficient phage to prevent the development of non-mucoid colonies. Twelve similar samples were taken from one of the tubes (Table 4). As with the Newcombe spread-plate experiments there was a suggestion that the results supported the random-mutant hypothesis. A larger experiment was carried out with a third strain, 1608, and the inoculum was allowed to grow over a longer period (8 hr.) before the samples were taken. The difference between the variance and the mean in this test seemed conclusive evidence in favour of the random mutation hypothesis.

Table 4. *Results of fluctuation tests*

Strain	Type	No. of samples	Samples from independent cultures				Samples from single cultures			
			Mean	Variance	χ^2	<i>P</i>	Mean	Variance	χ^2	<i>P</i>
T5 B	5	12	1.33	2.6	21.6	<0.05	0.67	0.66	.	.
SF40	27	12	3.75	6.39	18.7	<0.05	1.75	2.02	.	.
1608	12	39	11.1	64.4	2381.3	<0.001	4.1	1.2	45.7	>0.05

Several attempts to demonstrate mucoid variants in a non-mucoid culture by the replica plating technique of Lederberg & Lederberg (1952) were unsuccessful.

Some of the rather indefinite results obtained in these tests could be explained if the mucoid variants were unstable and tended to revert to the non-mucoid form. However, in numerous subcultures on plates and through

broth this has not been observed. At the same time, the mucoid variants grow more slowly than their non-mucoid parents. Fig. 1 shows the growth rate in broth of a mucoid variant of strain 1130 obtained by mouse passage, and that of the non-mucoid parent strain. The non-mucoid strain had an advantage in mixed culture which would be enough to keep the mucoid variants down to insignificant proportions in a series of laboratory subcultures.

The fact that the streptococci grow in chains which are not easily broken up on plating may also have influenced these results. Thus multiple mutant cells in one chain can probably only give rise to single mucoid colonies, leading to

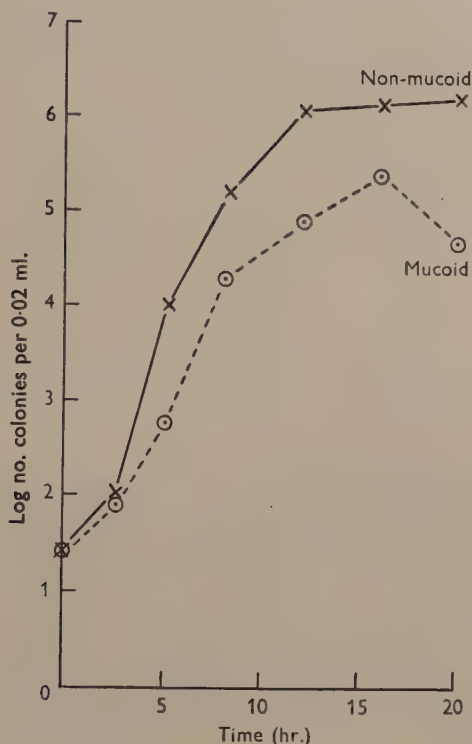


Fig. 1. Growth rate of non-mucoid and mucoid variants of strain 1130.

a reduction in the variance observed in the fluctuation and spread-plate experiments. The mean chain length of the cultures such as were used for these experiments was 7 with a range of 3 to 17.

M antigen in phage-passaged strains

Apart from their colony form, the phage-surviving variants may also differ from their parents in M antigen content, as judged by the amount of visible precipitate formed when an acid extract is mixed with an equal volume of specific antiserum in a capillary tube.

The M antigen was increased in 9 of the 10 strains most closely examined for this change (Table 5). The most striking results were with strains which had given the merest trace of M when first tested. Their phage survivors gave very strong M reactions and the extracts could often be diluted to 1/64. Even strains that had average amounts of the antigen often showed a marked increase in the amounts extracted from the phage survivors, although not all such strains became mucoid. A number of glossy variants containing no M substance were passaged through broth to which phage had been added, and though they became mucoid they showed no trace of M antigen.

Table 5. *Amount of M antigen extracted from parent strains and their phage survivors*

Strain	Type	Parent strain		Phage survivors	
		Final active dilution of extract	Colony appearance	Final active dilution of extract	Colony appearance
Richards	3	1/8	Mucoid	1/64	Mucoid
T5 B	5	1/32	Non-mucoid	1/64	Mucoid
S43	6	1/8	Mucoid	1/32	Mucoid
927	12	1/16	Non-mucoid	1/64	Non-mucoid
1518	12	1/16	Non-mucoid	1/64	Mucoid
1130 matt	12	1/16	Non-mucoid	1/64	Mucoid
1130 glossy	12	Nil	Non-mucoid	Nil	Mucoid
J.L.	12	Trace undiluted	Non-mucoid	1/16	Mucoid
1506	30	Trace undiluted	Mixed	1/16	Mucoid

Again, the process seemed to be one of selection, since in no instance did we succeed in promoting M antigen production by strains that failed to show any trace of it in the initial test, but the reason for the selection is not clear.

Table 6. *The effect of trypsin on the phage resistance of matt M-containing variants*

Bacterial lawn from broth culture of	Lysis on plates spread with specified lawn and inoculated with phage diluted				
	Nil	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴
1130 matt	CL	CL	+++	—	—
1130 glossy	CL	CL	CL	++	50
1130 matt + trypsin	CL	CL	CL	++	40
1130 glossy + trypsin	CL	CL	CL	++	70

CL = confluent lysis; +++ = lysis almost complete; ++ = 50 % lysis; + = 10–50 plaques; ± = fewer than 10 plaques.

The phage was titrated on a solid medium using either the matt-colony M-containing variant or the glossy variant, without M antigen, for the lawn, and the matt strain seemed more resistant to the phage. Both variants were then grown in broth containing active trypsin to destroy the M antigen, and the titration was repeated on trypsin-containing plates (Table 6). The cultures

gave identical phage titres. The increase in phage action on the matt M-containing strain when it was grown in the presence of trypsin may have been due to the destruction of the M antigen, but it might also have been due to the very noticeable improvement in the smoothness of the bacterial lawn.

Using these matt and glossy variants of strain 1130, viable counts were made of the survivors present after incubation for 24 hr. in broth containing phage, with and without the addition of trypsin. The matt-colony culture yielded 5.3×10^5 viable organisms in the absence of trypsin and 1.8×10^3 in its presence; for the glossy-colony culture the counts were respectively 2.3×10^5 and 3.0×10^4 . However, this partial resistance to the action of phage by the matt-colony M-containing variants may well have been due simply to their extreme granularity in fluid and on solid media. That it was not due to M antigen was also suggested by the results obtained when the parent strain of a weak M-positive culture was phage-passaged through broth with the addition of active trypsin to ensure that little or no M antigen was produced during growth. The survivors of three such subcultures, when grown in trypsin-free medium and extracted, gave equally good strongly positive M precipitation reactions as when phage passage was through broth without the addition of trypsin.

T antigens of phage-passaged strains

The T antigens of group A streptococci are most easily identified by slide agglutination trypsin-digested suspensions. The development of the antigen is favoured by growth at 30°. T agglutination was tested on cultures of phage survivors from a variety of types, grown at 30 and 37°.

Phage survivors grown at either temperature were usually inagglutinable when smooth enough to test without trypsin digestion. After trypsin treatment all the 30° suspensions reacted, although some very poorly, but a number of these grown at 37° still failed to agglutinate. Streptococci containing an M antigen not infrequently fail to agglutinate with T antisera, and the failure of most of the strains grown at either temperature to react before trypsin digestion is in agreement with results obtained when testing many naturally mucoid strains of streptococci. It is recognized that a hyaluronic acid capsule interferes with agglutination.

These results support those of Schwartzman (1927), who reported that a number of strains failed to agglutinate after surviving phage action. As the benefit of incubation at a lower temperature and the effect of trypsin digestion on the suspensions had not been described, these tests were presumably done on untreated 37° suspensions, and with unabsorbed sera.

Virulence of phage-selected cultures

Mouse virulence. Since an antigenic and colonial change might well be associated with an alteration in virulence, a number of phage survivors and their parent strains were tested for their mouse virulence. The parent strains had either (a) poor M content and mucoid colonies, (b) good M content and

Table 7. *Comparison of mouse virulence of strains before and after exposure to active phage*

Strain	Type	M antigen	Mucoid colony	Deaths among 10 mice, each receiving 0.5 ml. culture diluted						Approx. LD ₅₀ × 10 ³	
				Nil	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵		
Richards	3	Parent	+	+	nt	nt	4	1	0	0	220
		Phage survivor	+	+	nt	nt	10	10	4	1	
T5 B	5	Parent	+	+	0	0	0	nt	nt	nt	> 120,000
		Phage survivor	+	+	10	2	0	nt	nt	nt	
J.L.	12	Parent	±	•	10	4	1	1	0	nt	790
		Phage survivor	+	+	10	10	10	9	8	nt	
1130	12	Parent	+	•	10	7	3	2	nt	nt	2,200
		Phage survivor	+	+	10	10	10	8	nt	nt	
1506	30	Parent	+	•	nt	nt	8	8	4	1	25
		Phage survivor	+	+	nt	nt	10	10	9	9	

nt = not tested.

• • = no mucoid colonies.

• = no mucoid colonies. nt = not tested.

non-mucoid colonies, or (c) good M content and mucoid colonies; the last group were already partially virulent (Table 7). There was an increase in the mouse virulence of all the phage survivor strains, including 'Richards' which was already mucoid and M-reactive.

The promotion of virulence by *in vitro* cultivation of streptococci in such a selective system has not hitherto been described, so that it seemed worth investigating rather more closely the variations within one type.

From the non-mucoid type 12 strain 1130, matt and glossy variants were obtained—the matt having M antigen and the glossy having none. After exposure to phage, grossly mucoid variants were obtained from each strain, without changing the M antigen content. The four variants were tested for mouse virulence (Table 8). Clearly the matt mucoid variant was the most, and the non-mucoid glossy strain the least virulent. The parent, non-mucoid matt strain and the mucoid glossy seemed approximately equal in their lethal effect. It is interesting to note that in this instance the ability to produce hyaluronic acid enhanced virulence as much as the possession of M antigen.

Table 8. *Mouse-virulence tests on matt and glossy variants of strain 1130, before and after exposure to phage*

Colonial variant	M antigen	Mucoid colony	Deaths within 48 hr. among mice in each group of 10 receiving 0.5 ml. culture diluted						Approx. LD 50 × 10 ³
			Nil	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	
Glossy	—	—	9	1	0	0	0	—	11200
Glossy phage survivor	—	+	10	9	0	2	0	—	355
Matt	+	—	10	10	9	6	—	—	100
Matt phage survivor	++	+	10	10	10	10	10	10	0.6

Ability to grow in human blood. Although most strains of group A streptococci isolated from acute cases of disease are avirulent for mice they are usually able to grow readily in normal human blood. A number of the parent strains and their phage survivors have been tested for growth in blood, since this might be a better guide to human virulence than the ability to kill mice. Some of the parent strains grew readily, but of those that did not do so, all gave phage-surviving variants which were able to grow (Table 9). The four variants of 1130 used in the mouse virulence tests were also tested in normal blood; only the matt mucoid strain was able to multiply. The possession of M substance alone or capsule material alone was not enough to ensure multiplication.

DISCUSSION

From the work described here it is clear that some active phages can lead to the development of a population of streptococci having two of the characteristics of the most virulent strains—the possession of M antigen and the ability to produce a hyaluronic acid capsule. Though it is accepted that there are other

features concerned in virulence, these two have been studied in some detail. Estimation of virulence is difficult, for it is known that strains apparently virulent for man are not necessarily virulent for mice and the converse may also be true. However, considering mouse virulence and the ability to overcome the bactericidal power of human blood together, it is reasonable to suppose that a strain at first avirulent by either standard which becomes virulent by both has undergone a change drastic enough to warrant some respect. While those working with *Corynebacterium diphtheriae* and its phages along the lines suggested by Freeman's original work (1951) suggest that the change may be an induced one (Groman, 1953; Barksdale & Pappenheimer, 1954), the change observed in streptococci seems most probably to be due to selection. The ease with which some strains can also be made mucoid by mouse passage, the fact that many strains initially yield mucoid and non-mucoid colonies, and also the experiments with multiple sampling, suggest that this must be so.

Table 9. *Survival of streptococci in human blood before and after exposure to phage*

Strain	Colony	M antigen	Growth from mixture sampled at:			
			3 hr.		20 hr.	
			A	B	A	B
Type 12	Control	Non-mucoid	trace	±	++	—
	Phage survivor	Mucoid	++	+	++	++++
Type 5	Control	Non-mucoid	+	—	++	++++
	Phage survivor	Mucoid	++	+	++++	++++
Type 30	Control	Mixed	trace	—	—	+
	Phage survivor	Mucoid	+	+	++	++++
1130 glossy, type 12	Control	Non-mucoid	—	±	—	—
	Phage survivor	Mucoid	—	±	—	—
1130 matt, type 12	Control	Non-mucoid	+	++	—	++
	Phage survivor	Mucoid	++	++	+	++++

A=inoculum, 0.02 ml. of culture diluted 10^{-5} ; B=inoculum, 0.02 ml. of culture diluted 10^{-3} ; —=no growth; ±=1 to 10 colonies; +=10 to 50 colonies; ++=50 to 200 colonies; +++=more than 200 colonies; ++++=continuous sheet of growth.

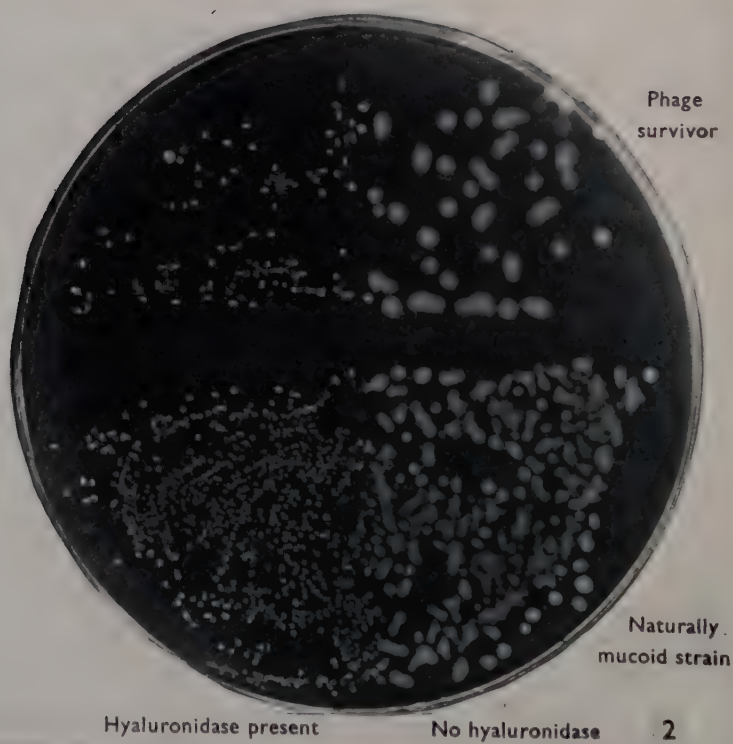
Whether a selective process of the same sort can take place *in vivo* is not known and this may repay further investigation. Evans (1933) stated that phage action did not take place in many body fluids, but it seems probable that the addition of hyaluronidase to a system such as she used might allow some lytic activity. It is of interest to recall that Evans, describing experiments where mice infected with group A streptococci were given phage filtrates in an attempt to save them, noted that the phage-treated mice died slightly more rapidly than the untreated controls.

The phages used here were lytic phages which originated from sewage and their true origin is somewhat obscure. None of the strains of streptococci passed through them seemed to become lysogenic, and streptococci recovered from mice infected with phage survivors were not lysogenic nor contaminated



Parent strain

Phage survivor



Phage
survivor

Naturally
mucoid strain

Hyaluronidase present

No hyaluronidase

2

with phage. The phage-selected mucoid streptococcal variants were stable when freed of phage, but whether they are stable for the M antigen for more than about six subcultures in the absence of phage was not tested.

I wish to thank Dr E. S. Anderson and Dr C. C. Spicer for their interest and advice.

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EXPLANATION OF PLATE

Fig. 1. Mucoid colonies of phage survivor, $\times 4$.

Fig. 2. Effect of hyaluronidase on colonies of naturally mucoid and phage-survivor mucoid strains. $\times 1$.

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Observations on the Carbohydrate Metabolism of the Flagellate *Strigomonas oncopelti*

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SUMMARY: Cultures of *Strigomonas oncopelti* multiplied more rapidly and a higher peak population was reached when glucose was added to the peptone medium. The rate of glucose consumption varied during the growth cycle; the maximum rate recorded was 90 $\mu\text{g.}/10^8$ organisms/24 hr. The breakdown of glucose resulted in the production of acetic, succinic, probably lactic, and an unidentified non-volatile acid.

Strigomonas oncopelti, a flagellate belonging to the family Trypanosomidae, is a parasite of latex plants and is transmitted by plant bugs. It can be readily cultivated in peptone water. Noguchi (1926) and Lwoff (1934) showed that the organism can metabolize glucose. The experimental work to be described shows the difference between the growth characteristics of *S. oncopelti* in the presence and absence of glucose, and demonstrates how the rate of glucose consumption varies during the growth cycle. The glucose breakdown and accompanying acid production are compared with those of other members of the family Trypanosomidae.

METHODS

Cultivation. Stock cultures of a bacteria-free strain of *Strigomonas oncopelti* received from Dr A. Lwoff were grown in 8 ml. volumes of 3% Oxoid peptone (Oxo Ltd.) at pH 7.4 containing 0.5% NaCl. The medium was sterilized by autoclaving at 15 lb./sq.in. for 20 min. The organisms were grown at room temperature and subcultures were made every 4-7 days. For the experimental work the organisms were grown in 3% Oxoid or Difco proteose peptone containing 0.5% NaCl.

Counting. The cultures were mixed gently before sampling as the organisms tended to form a film at the surface. Samples of 1 ml. were fixed with a drop of formalin. The organisms adhered together in rosettes in the cultures and the fixed samples were therefore shaken vigorously before counting in order to break up the clumps. Duplicate counts were made on each sample in a Neubauer haemocytometer, a minimum of 300 organisms being counted, and the mean of the results then taken.

pH measurement. A Cambridge bench type pH meter (Cambridge Instrument Co. Ltd.) was used which was standardized with buffers of pH 4.0 and 9.2.

Glucose estimation. Glucose was estimated by the method of Somogyi (1937).

A sample of 1 ml. of medium was diluted with 10 ml. of water and deproteinized by adding 1 ml. of each of 7% (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and of 10% (w/v) sodium tungstate. After removal of the precipitate by centrifugation 1 ml. of the supernatant fluid was diluted with 4 ml. of water before addition of the copper reagent. Duplicate determinations were carried out on each sample.

Extraction of organic acids from the medium. The medium (165 ml.) was adjusted to pH 1.0–1.5 with 5N- H_2SO_4 and the organic acids extracted with ether in a continuous extraction apparatus. The ethereal extract was shaken with a slight excess of alkali (0.1N-NaOH) and the aqueous solution containing the acids was concentrated to approximately 25 ml. The steam-volatile and non-volatile acids were separated by steam distillation of the acidified solution (pH 1.0–1.5) and the distillate was titrated under CO_2 -free conditions, using phenolphthalein as indicator. The neutralized distillate was concentrated and kept for chromatographic analysis.

In order to avoid the presence of large amounts of inorganic salt the non-volatile acids were re-extracted with ether from the solution remaining after steam distillation, and finally obtained as their sodium salts by shaking the ether with the minimum amount of 0.02N-NaOH required for complete extraction and then evaporating the solution to dryness.

Analysis of volatile fatty acids. The volatile fatty acids were analysed by gas/liquid partition chromatography (James & Martin, 1952).

Analysis of non-volatile acids. The non-volatile acids were examined by paper chromatography using the general technique of Long, Quayle & Stedman (1951). The following solvent systems were used: (1) ethanol 80 vol. + aqueous ammonia, 0.88 sp.gr., 4 vol. + water 16 vol.; (2) ethanol 90 vol. + aqueous ammonia, 0.88 sp.gr., 5 vol. + water 5 vol. In some instances the papers were sprayed with the indicator recommended by Duncan & Porteous (1953).

EXPERIMENTAL

Growth of Strigomonas oncopelti in the presence and absence of glucose

Two growth curves were constructed for *Strigomonas oncopelti*. For the first the organism was grown in peptone (Difco proteose) water alone and for the second in peptone water + 0.5% glucose. The peptone medium without added glucose contained the equivalent of 25 mg. reducing sugar/100 ml. In both instances the procedure was as follows. The medium (500 ml.) was sterilized in a flask. As controls, two samples of 8 ml. were withdrawn before inoculation. The pH value and glucose content of one sample were determined at the beginning of the experiment. The second sample was incubated with the inoculated medium, the pH value and glucose content being estimated at the end of the experiment. The flask of medium was inoculated with a 5-day culture of *S. oncopelti* to give an initial count of *c.* 500,000 organisms/ml. The inoculum had been grown in peptone water without added glucose. After inoculation the medium was distributed into test-tubes in 8 ml. volumes. Immediately afterwards four tubes were withdrawn for counting and for pH

and glucose estimations. The remaining tubes were left at room temperature (*c.* 20°) in the dark. Four tubes were withdrawn daily until the organisms began to die. From each tube 1 ml. was fixed for counting and 3 ml. used for the pH estimation. The remainder of the culture was then centrifuged and the supernatant stored at -10° for subsequent glucose estimations.

The results of the pH measurements and the counts are illustrated in Figs. 1 and 2; each value used is the average for the four parallel tubes. In the absence of glucose (Fig. 1) the peak population of *c.* 25×10^6 organisms/ml. was reached on the eighth day. This represented 5.3 generations; during the most rapid period of multiplication the generation time was 14 hr. By the ninth day some of the organisms had died and the counts were discontinued.

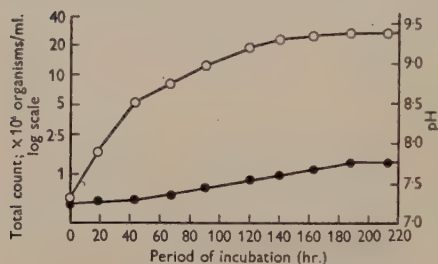


Fig. 1

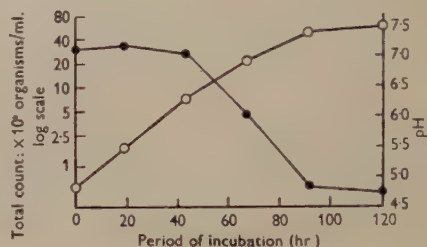


Fig. 2

Fig. 1. Growth and pH curve of *S. oncopelti* in the absence of glucose. ○—○, growth; ●—●, pH curve.

Fig. 2. Growth and pH curve of *S. oncopelti* in the presence of glucose. ○—○, growth; ●—●, pH curve.

When 0.5% glucose was present in the medium the growth curve was different from that obtained without glucose (Fig. 2); the organisms multiplied more rapidly, the shortest generation time was 12 hr., a higher peak population was reached and the cultures began to die sooner. The peak population of 55×10^6 organisms/ml. occurred on the fifth day; this represented 6.5 generations. Dead organisms then began to appear in considerable numbers and the counts were discontinued.

It appeared, therefore, that although glucose was not an essential constituent of the medium it increased the rate of multiplication and a higher peak population was reached in its presence. The earlier death of the cultures in glucose peptone may possibly be associated with the low pH value of the medium resulting from the breakdown of glucose. When glucose was not added to the medium the pH value increased steadily from 7.3, reaching 7.8 on the eighth day (Fig. 1). The pH value of the medium containing 0.5% glucose fell from 7.2 to 4.7 by the fifth day (Fig. 2). After the organisms began to die the pH value rose again slowly and reached pH 5.1 on the tenth day. During the growth of the cultures the pH value of the uninoculated control remained constant.

Glucose consumption

Glucose estimations were carried out on one of the four tubes withdrawn each day. During the period of the experiment the apparent reducing-sugar content of the uninoculated control increased by 40 mg./100 ml., presumably due to concentration by evaporation. The rate of glucose consumption calculated from the glucose estimations is shown in Fig. 3. The rate of glucose consumption was calculated from the mean of the counts at the beginning and end of each 24 hr. period and the quantity of glucose which disappeared from the medium during the period. The rate of glucose consumption was not constant throughout the growth cycle (Fig. 3), but increased while the organisms were multiplying rapidly and decreased rapidly as the division rate slowed down. By 120 hr., when the population had reached 55×10^6 organisms/ml. and the pH value had fallen to 4.7, only 25 mg. glucose/100 ml. was left in the medium. The maximum rate of glucose consumption recorded was $90 \mu\text{g.}/10^6$ organisms/24 hr. This represented a ninefold increase in rate during the time the organisms were multiplying.

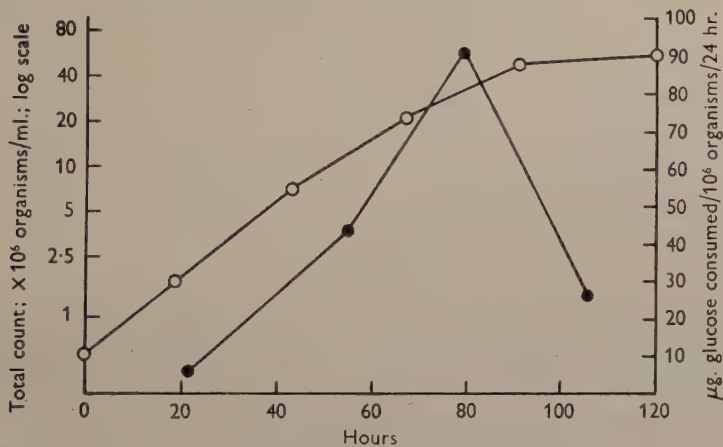


Fig. 3. Growth and glucose consumption of *S. oncopelti*.
 ○—○, growth; ●—●, glucose consumption.

The acids produced from the breakdown of glucose

Glucose peptone (Oxoid) medium (250 ml.) was inoculated with a 5-day culture of *Strigomonas oncopelti* to give an initial count of *c.* 500,000 organisms/ml. and was left at room temperature (*c.* 20°) in the dark for 4 days; an equal volume of uninoculated glucose peptone was left for the same period as a control. After growth the organisms were separated by centrifugation and the supernatant fluid was clarified with a Seitz filter. Samples of the volatile and non-volatile organic acids present in both uninoculated and used media were prepared as previously described. Examination of these samples showed an increase of both volatile and non-volatile acids in the used medium. The increase in volatile acid was entirely due to an increase in acetate (Table 1).

Examination of the samples of non-volatile acids by paper chromatography showed that lactic acid was present in both uninoculated and used medium, while succinic acid and another unidentified acid were present in used medium only. The unidentified spot was not any of the following: oxalic, citric, tartaric, malonic, malic, maleic, adipic, pimelic, pyruvic, glutaric, glycolic, fumaric, crotonic, acrylic or β -hydroxybutyric acids. In earlier experiments the non-volatile acid samples were examined for lactic acid by the method of Friedemann & Graeser (1933); this method is not specific for lactic acid, but the large apparent increase (threefold) of lactic acid in the used medium strongly suggests that it was produced as an end product. The breakdown of glucose by *Strigomonas oncopelti* results, therefore, in the production of acetic, succinic, and probably lactic acids, and an unidentified non-volatile acid.

Table 1. Volatile fatty acid content of glucose peptone medium.
(1) Uninoculated, (2) after growth of *Strigomonas oncopelti*

	(1) Uninoculated medium		(2) Used medium	
	m-mole/l.	%	m-mole/l.	%
Formate	0.6	21	0.7	12
Acetate	2.1	71	4.7	83
Propionate	0.1	4	0.1	2
Butyrate	0.1	4	0.1	2
Valerate	0	0	Trace	1
Total	2.9	100	5.6	100

DISCUSSION

The carbohydrate metabolism of cultivated forms (invertebrate host stage) of trypanosomes and of other members of the family which have been grown *in vitro* has not been studied as thoroughly as that of the blood stream forms of trypanosomes (from the vertebrate host). However, the carbohydrate metabolism of *Strigomonas oncopelti* shows some similarities to that of the other members of the Trypanosomidae grown *in vitro*. Some of the species which have been studied are not dependent on a supply of glucose. *Trypanosoma cruzi* (von Brand, Tobie, Kissling & Adams, 1949), *T. gambiense* and *T. rhodesiense* (Tobie, von Brand & Mehlman, 1950) have been grown without added glucose, although glucose was consumed when it was available. It is interesting that glucose consumption by the blood stream form of *T. cruzi* cannot be detected (von Brand *et al.* 1949) while the blood stream forms of *T. gambiense* and *T. rhodesiense* have a much higher rate of consumption than the cultivated forms (von Brand & Tobie, 1948). *Strigomonas media* and *S. parva* have been grown in peptone medium without added glucose (Lwoff, 1936), and *Leptomonas ctenocephali* did not utilize glucose under aerobic conditions even when it was present (Lwoff, 1934). Other organisms which have been cultivated *in vitro* and shown to consume glucose include several species of *Leishmania* (Salle & Schmidt, 1928; Chang, 1948).

The rate of glucose consumption by *Strigomonas oncopelti* varies considerably during the growth cycle, the rate increasing while the generation time is short, and falling rapidly when multiplication ceases; Fulton & Joyner (1949) found similarly that young cultures of *Leishmania donovani* used more glucose than old cultures.

The incomplete oxidation of glucose which results in the production of a number of organic acids appears to be characteristic of the members of the Trypanosomidae so far investigated. *Strigomonas oncopelti* produced acetic, succinic and probably lactic acids, and also an unidentified non-volatile acid. Formic, acetic, pyruvic, lactic, oxalic and succinic acids have been identified as end products of glucose breakdown by various trypanosomes and leishmanias (von Brand, 1951); the unidentified acid produced by *S. oncopelti* is not one of these. As *S. oncopelti* is a parasite of plants it might well be expected to show some different characteristics from the parasites of vertebrates.

Most of the work was carried out during the tenure of a studentship from the Medical Research Council. I should like to thank Dr Muriel Robertson, F.R.S., for her guidance and help, and I am grateful to Dr E. F. Annison for analysing the volatile fatty acids and for the lactic acid estimations.

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The Preservation of Lactobacilli by Freeze-drying

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SUMMARY: In developing a technique for the freeze-drying of lactobacilli particular attention was paid to the percentage survival rate; this was estimated by viable counts immediately after drying and after storage of the dried cultures. The main factor influencing survival rate was the suspending medium, the best results being obtained in horse serum with 8 % added glucose. Forty-four cultures, fourteen freshly isolated, representative of a comprehensive collection of 452 lactobacilli (see Briggs, 1953*a*) were freeze-dried in this vehicle using the apparatus and technique described. The majority of the survival rates were over 50 % immediately after drying, and over 25 % after 6 months storage; all but two of the freshly isolated strains dried well. Some of the dried cultures were tested again after a storage period of about 2 years and then showed satisfactory percentage survivals.

For taxonomic studies of 452 strains of lactobacilli (Briggs, 1953*a*) cultures were maintained in yeast glucose litmus milk plus chalk at 4°, which, although satisfactory for survival, involved considerable expenditure of media and labour; a less cumbersome method of maintenance was required, and the possibilities of freeze-drying were investigated.

Since no outstanding method for lactobacilli had been described, the technique which had proved satisfactory for the freeze-drying of streptococci at this Institute was examined. The effects of different methods of preparing the culture, of various suspending media and of other modifications of the freeze-drying technique were studied by determining the percentage survivals immediately after drying and at intervals during storage. A freeze-drying technique was evolved using as test organisms strains of lactobacilli particularly sensitive to drying. The method was then applied to forty-four cultures representative of the eight groups of Briggs (1953*a*), including fourteen freshly isolated strains.

METHODS

In studying the conditions affecting the viability of lactobacilli during freeze-drying and subsequent storage, the different factors involved were varied one by one. Deviations from, or comparisons with, the standard method described below are recorded in the appropriate places.

Apparatus. The apparatus, illustrated in Pl. 1, was similar to Swift's (1937) and also to that used by Proom & Hemmons (1949), the chief components being an efficient vacuum pump ('Speedivac', W. Edwards and Co. (London) Ltd.), a desiccator and a large lagged box. Vacuum in the system was determined by means of an Edwards's 'Vacustat', and dry air was introduced as required through a column of silica gel. A manifold, taking ten tubes, was

connected to the evacuating system and an Edwards's high frequency vacuum tester was used for the detection of vacuum in the tubes on the manifold and in the sealed tubes.

Standard drying technique. Cultures were incubated for 24 hr. anaerobically on slopes of 10 ml. tomato glucose Tween agar (T.G.T.A.; Briggs, 1953*b*). 0.5 ml. of separated milk + 3 % lactose was added to each tube and the growth washed off. The suspensions were bulked, thoroughly mixed and 0.1 ml., accurately measured in special pipettes, introduced into each drying tube, care being taken to deposit all the suspension in the bottom; 0.1 ml. quantities of suspension were also added to duplicate tubes of 10 ml. Ringer's solution for dilution and plating to give an estimate of the original viable count.

The drying tubes were placed in a dish of powdered solid CO₂ for 3 min. and then transferred to the desiccator which contained a basket to hold 400 tubes, and a large dish of P₂O₅. Solid CO₂ had been stored in the lagged box overnight to cool it. The desiccator was placed in the box, connected to the evacuating system and the pump started. The pump was left running overnight (18 hr.), the vacuum reaching at least 0.01 mm. of mercury. After the pump had been stopped dry air was introduced slowly and the tubes removed from the desiccator. The cotton-wool plugs were pushed half-way down the tubes and the glass constricted above the level of the plugs. The tubes were then evacuated on the manifold and sealed under vacuum. The dried cultures were stored at room temperature in the dark. Duplicate tubes required for estimating the viable count immediately after freeze-drying were not sealed but were reconstituted as soon as they were removed from the desiccator.

Reconstitution. Tubes were tested for vacuum, opened, and 0.1 ml. of sterile distilled water at room temperature was added to each tube. The contents of each tube were then transferred to 10 ml. Ringer's solution, the tube being washed out once with this solution.

Viable counts. The viable count before freeze-drying was estimated from the suspension to be dried. Tenfold dilutions were made in Ringer's solution and duplicate plates prepared for each dilution tested. To avoid the use of large numbers of anaerobic jars plates were prepared with double layers of agar, the lower consisting of T.G.T.A. containing 0.002 M-sodium mercaptoacetate and the bacterial suspension; this was covered with a layer of nutrient agar also containing 0.002 M-sodium mercaptoacetate. This method of plating proved satisfactory for all the species of lactobacilli used in the early work with the exception of *Lactobacillus bulgaricus*: for this species, and for a few others in later studies (Table 5) T.G.T.A. + mercaptoacetate plates were not covered with the upper layer of agar but were incubated in anaerobic jars. All plates were incubated for 3 days at the optimum temperature of the culture under test before being counted.

Organisms. The cultures used in this work were those studied by Briggs (1953*a*). In preliminary tests strains of *Lactobacillus helveticus*, *L. casei* and *L. brevis* gave higher percentage survivals than *L. acidophilus* or *L. bulgaricus*. Therefore most of the early work was done with one strain of *L. acidophilus*—A1 (Briggs, 1953*a*).

RESULTS

Effect of the nature of the suspending medium

Separated milk + 3 % lactose had been used successfully for several years at this Institute for the freeze-drying of streptococci and was adopted for the lactobacilli as a standard. Three other suspending media were tested: horse serum, horse serum + 8 % glucose and Naylor & Smith's (1946) reducing medium. 8 % concentration of glucose in horse serum was chosen so that the total sugar in the serum + glucose suspending medium approximated that in the milk + lactose medium. Preliminary experiments showed that the immediate survival of *Lactobacillus acidophilus* A1 in serum alone was approximately 75 % of that obtained in the standard milk + lactose medium; consequently serum alone was not tested further. Table 1 summarizes the results of experiments in which the numbers of viable organisms after freeze-drying in the standard milk medium were compared with those in serum + glucose and in the reducing medium.

Table 1. *Effect of different suspending media on the survival of freeze-dried lactobacilli*

Strain	Suspending medium*	Percentage survival after storage time							
		Weeks				Months			
		0†	1	2	3	1	3	6	21
<i>L. acidophilus</i> A1‡	A	5.1	nt	nt	8.4	nt	28.6	14.8	nt
	B	14.4	nt	nt	12.5	nt	35.5	35.6	nt
<i>L. acidophilus</i> A1‡	A	36.0	29.8	nt	nt	5.3	1.9	6.0	nt
	C	10.0	2.0	nt	nt	0.3	0.5	1.4	nt
<i>L. acidophilus</i> A6	A	3.9	nt	1.2	nt	2.0	nt	nt	nt
	B	2.5	nt	4.8	nt	2.3	2.6	nt	nt
<i>L. bulgaricus</i> B2	A	22.5	nt	12.4	nt	23.1	21.4	11.8	7.7
	B	18.0	nt	18.7	nt	17.2	23.8	16.5	14.5
	C	2.3	nt	0.6	nt	0.04	0.7	0.5	0.03
<i>L. pentoaceticus</i> E2	A	14.9	nt	23.2	nt	6.7	5.0	nt	nt
	B	67.2	nt	23.9	nt	35.7	18.2	nt	nt
<i>L. casei</i> H1	A	82.9	nt	60.5	nt	56.5	31.8	nt	nt
	B	77.6	nt	71.1	nt	77.6	45.2	nt	nt

* A: separated milk + 3 % lactose; B: horse serum + 8 % glucose; C: Naylor & Smith's (1946) reducing medium.

† Immediately after freeze-drying.

‡ Results of separate experiments.

nt = not tested.

Despite variations between the results of replicate experiments the data in Table 1 show that the reducing medium (C) gave considerably lower survival rates than either of the other suspending media. Serum + glucose gave more satisfactory survivals than milk + lactose. Fig. 1, in which the log. of the viable count has been plotted against time, illustrates the results obtained with *Lactobacillus bulgaricus* B2 in the milk and serum suspending media. As the storage time increased, the loss of viability in serum + glucose was less than in milk + lactose.

The superiority of the serum + glucose suspending medium in these experiments warranted its use in further studies, described later, with representative strains of lactobacilli.

Effect of reconstitution temperature

Six tubes of *Lactobacillus acidophilus* A1 which had been freeze-dried by the standard method (p. 504) were opened, and three reconstituted with sterile distilled water at room temperature and three with water at 50°. The former showed an average count of 4.8×10^6 /tube (0.1 ml.), corresponding to 40 % survival of the original, whilst those reconstituted at 50° showed a count of 3.6×10^6 /tube, representing 30 % survival; these results are in agreement with those of Speck & Myers (1946). Henceforth, all tubes were reconstituted with water at room temperature.

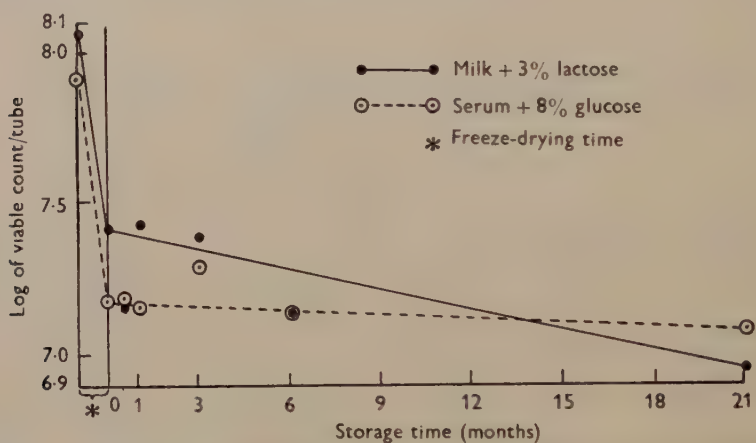


Fig. 1. Survival of *Lactobacillus bulgaricus* B2 after freeze-drying and storage in two suspending media.

Effect of different methods of preparing the cultures

Since the large quantities of acid which some strains of lactobacilli produce might be detrimental to the organisms during both freeze-drying and storage, it was anticipated that cultures grown on slopes would be more suitable for drying than organisms from broth, in which some strains can produce a pH value of 3.5 or less in 24 hr. *Lactobacillus acidophilus* (A1) was grown on T.G.T.A. slopes and in tomato glucose Tween broth (T.G.T.B.) incubated both aerobically and anaerobically for 24 and 48 hr. Contrary to expectation, the results immediately after freeze-drying showed that the cultures from broth, particularly those incubated anaerobically for 24 hr., gave higher survival rates than those from agar (Exp. 1, Table 2).

Three further experiments were made in which the standard method (growth on agar slopes for 24 hr. anaerobically) was compared with preparation of the culture by anaerobic incubation of broth for 24 hr. Table 2, which

includes data covering an 18 months storage period, shows the variable results obtained, the different methods of preparing the culture resulting in similar percentage survivals. In these experiments the broth cultures were centrifuged and the cells taken up directly in the suspending medium; in further studies the centrifuged cells were washed twice in 0.85 % NaCl to remove any free acid before resuspension. No difference was found between the percentage survivals of washed and unwashed cells.

Table 2. *Effect of growth medium on the survival of freeze-dried cultures of Lactobacillus acidophilus A1*

Exp.	Medium*	Percentage survival after storage time, months			
		0†	1	3	18
1	A	48.2	45.3	41.7	nt
	B	80.2	31.7	40.3	16.0
2	A	71.2	37.5	43.5	17.9
	B	51.7	20.6	46.9	29.9
3	A	68.5	66.3	57.4	32.8
	B	50.3	67.4	50.0	31.9
4	A	59.0	59.6	66.6	nt
	B	60.0	51.9	71.3	nt

* A: T.G.T.A. (Briggs, 1953*b*) slope incubated anaerobically for 24 hr. (standard method).

B: T.G.T.B. (Briggs, 1953*b*) incubated anaerobically for 24 hr.

† Immediately after freeze-drying.

nt = not tested.

Effect of variation of the original count

The original counts of strains of *Lactobacillus helveticus*, *L. casei* and *L. brevis* were between 1.0×10^8 and 1.0×10^9 /tube (0.1 ml.); strains of *L. acidophilus* and *L. bulgaricus* had lower original counts (between 1.0×10^7 and 1.0×10^8 /tube) and also showed lower percentage survivals. The effect of variation of the original count was studied by freeze-drying suspensions, together with their 1/10, 1/100 and 1/1000 dilutions in milk + lactose. The immediate percentage survivals of two strains, *L. acidophilus* A1 and *L. casei* C14, are shown in Table 3; the figures after storage were not determined.

Table 3. *Effect of suspension density on the survival of freeze-dried lactobacilli*

Strain	Dilution of original suspension	Original average count per tube (0.1 ml.)	Percentage survival immediately after freeze-drying
<i>L. acidophilus</i> A1	0	12,625,000	63.8
	1/10	887,500	95.6
	1/100	132,500	65.5
	1/1000	20,725	66.2
<i>L. casei</i> C14	0	138,250,000	63.5
	1/10	13,580,000	85.4
	1/100	1,670,000	80.3
	1/1000	205,250	78.0

A 1/10 dilution of the original suspension, irrespective of the number of organisms present, gave the highest percentage survival; these observations are discussed on p. 509.

The separate effects of freezing, drying and storage

It was of interest to determine the separate contributions to the total lethal effect of freezing, drying and storage. The results shown in Tables 1 and 2 and in Fig. 1 show that a much greater loss of viability occurred during the actual freeze-drying process than during storage. Table 4A shows the results with two freeze-dried strains that had been stored for almost 2 years. Although the percentage survivals fell during storage, more than 8% of the original viable organisms were still alive after 22 months; this corresponded to a count of 15×10^6 organisms/tube (0.1 ml.) of *Lactobacillus brevis* X1 and 22×10^6 of *L. helveticus* H5.

The percentage survivals of two strains of *Lactobacillus acidophilus* were observed after freezing in CO₂-ice for 3 min. (see p. 504), and also after the freeze-drying process had been completed (Table 4B); the greater proportion of organisms was killed during the drying stage. The percentage survival figures after 1, 3 and 18 months storage have been included in the table to illustrate the relative loss of viability during freezing, during the complete freeze-drying process and during storage.

Table 4. *Survival of lactobacilli after freezing, freeze-drying and storage*

		Percentage survival						
Strain		After freezing only	Immediately after freeze-drying	After storage time, months				
				$\frac{1}{2}$	1	3	6	18 22
A	<i>L. brevis</i> X1	nt	56.7	39.2	37.1	51.3	23.9	nt 8.2
	<i>L. helveticus</i> H5	nt	39.4	31.6	32.9	26.3	23.6	nt 8.7
B	<i>L. acidophilus</i> A1	92.9	48.9	nt	50.8	41.2	nt	20.5 nt
	<i>L. acidophilus</i> A2	90.0	29.1	nt	25.1	32.3	nt	22.2 nt

nt = not tested.

Application of technique to representative cultures

Forty-four lactobacilli, fourteen freshly isolated, representative of Briggs's (1953a) classification were freeze-dried using the 'standard' method described on p. 504, save that 24 hr. cultures were suspended in horse serum + 8% glucose. The results, with some replicates, are shown in Table 5, together with the survival rates after 6 months storage. The growth temperatures used for the strains in each group and section (Briggs, 1953a) are also shown, as well as the different plating techniques for the viable counts.

The data in Table 5 show that survival rate varied with species, as Proom & Hemmons (1949) and Rhodes & Fisher (1950) found with other organisms; strain differences were not so marked as in Record & Taylor's (1953) work with

Escherichia coli. All the freshly isolated cultures gave high survival rates save for two in group V; however, most of the strains in this group dried relatively poorly. Further study of group V, for example with the organisms grown at a temperature lower than 45°, might prove profitable.

The indicated survival rate of 266·7 % achieved by *Lactobacillus buchneri* BC1 after 6 months storage was remarkable, but the figure of 286·7 % was obtained when a duplicate tube was counted. *L. buchneri* BC1 was a small coccobacillus and dispersal of clumped organisms may account for the figures obtained.

Distribution of the results of the fifty-two tests shown in Table 5 among arbitrary divisions of the survival rate range 0 to 100 + % gave the figures shown in Table 6. The majority of the survival rates were over 50 % immediately after drying, and over 25 % after 6 months storage.

DISCUSSION

Studies of the effects on bacteria of freeze-drying are, necessarily, of long duration; the work described here is not, therefore, complete though some cultures have been dried now for over 2 years. Moreover, these investigations are not comprehensive since they represent simply an attempt to solve the problem of maintenance of stock cultures of lactobacilli; studies of the more fundamental principles and factors involved have been deliberately avoided. Particular regard has been paid to percentage survival; the total number of organisms surviving is less important than the percentage, for it is the latter that indicates the degree of selection that may have occurred. The chief factor influencing the survival rate was the suspending medium; horse serum + 8 % glucose gave satisfactory results and was used in the routine drying of representative lactobacilli. The concentration (8 %) of glucose chosen, for the reason stated on p. 505, closely resembled that (7·5 %) with which Fry & Greaves (1951) obtained their best results with a paracolon bacillus.

Differences in the medium on which the cultures were grown had little effect on the counts obtained after freeze-drying, likewise the age of the culture (between 24 and 48 hr.). However, the numbers of organisms influenced the percentage survival in that a 1/10 dilution of the original suspension, irrespective of its concentration, gave the highest number of viable organisms immediately after drying. Yet this dilution corresponded to a count before drying of 1×10^6 /tube with one strain of *Lactobacillus* and 13×10^6 with another. In this connexion the work of Record & Taylor (1953) is of interest. With strains of *Escherichia coli* they found that 'a well-defined relationship existed between the concentration of organisms in the suspension before drying and the percentage of viable survivors recovered after reconstituting the dried product; the more dilute the suspension the lower the percentage of organisms surviving. . . . The dependence of percentage survival on the concentration of organisms in the suspension to be dried was shown to be due to soluble material derived from the organisms themselves.' Studies of this aspect with the lactobacilli would be of interest.

Table 5. *Survival of forty-four representative lactobacilli after freeze-drying in horse serum + 8 % glucose*

Strains, in groups and sections (see Briggs, 1953 <i>a</i>)	Percentage survival after 6 months storage		Incubation temperature	Technique for viable count
	0*	6		
Group I				
<i>L. acidophilus</i> A1	14.4	31.0	37°	(a)
<i>L. acidophilus</i> A1†	60.7	26.1		
<i>L. acidophilus</i> A20	7.4	29.0		
<i>L. acidophilus</i> A20†	50.0	13.9		
<i>L. acidophilus</i> A3	27.0	102.2		
<i>L. acidophilus</i> A4	30.7	7.4		
<i>L. acidophilus</i> A16	66.4	106.2		
<i>L. acidophilus</i> BF 4‡	55.3	24.6		
<i>Lactobacillus</i> PF 3‡	96.2	122.1		
Group II				
<i>L. bulgaricus</i> B2	18.0	16.5	37°	(b)
<i>L. bulgaricus</i> B2†	19.9	7.5		
<i>L. acidophilus</i> A18	56.6	31.3		
<i>L. bulgaricus</i> Y48	38.2	7.1		
<i>L. bulgaricus</i> SM10‡	46.8	17.6		
Group III				
<i>L. bulgaricus</i> B14	55.7	43.5	37°	(b)
<i>Lactobacillus</i> PR 8‡	86.0	115.5		
Group IV				
<i>L. helveticus</i> H5	39.4	23.6	37°	(a)
<i>L. helveticus</i> C10	117.4	96.6		
<i>L. casei helveticus</i> O9‡	47.3	90.2		
<i>L. casei helveticus</i> O9‡†	63.7	28.7		
<i>L. helveticus</i> YOG 3‡	93.8	15.3		
Group V				
<i>L. lactis</i> L1	9.5	0.2	45°	(a)
<i>L. lactis</i> L1‡	1.2	0.0007		
<i>L. thermophilus</i> THI	63.6	82.2	(but L1 at 37°) (but THI (b))	(b)
<i>L. leichmannii</i> LE2	20.1	0.5		
<i>L. leichmannii</i> LE2†	69.3	14.1		
<i>L. leichmannii</i> LE6	18.6	3.7		
<i>L. leichmannii</i> LE6†	8.4	1.6		
<i>L. lactis</i> AH7‡	6.0	1.7		
<i>L. lactis</i> AH7‡†	7.3	2.4		
<i>Lactobacillus</i> CS7‡	81.5	7.3		
Group VI				
<i>L. casei</i> C5	72.7	84.3	30°	(a)
<i>L. casei</i> C24	80.2	72.8		
<i>L. plantarum</i> P4	69.0	14.9		
<i>L. casei</i> H1	77.6	45.2		
<i>L. plantarum</i> SL5‡	69.6	38.4		

Table 5 (cont.)

Strains, in groups and sections (see Briggs, 1953 <i>a</i>)	Percentage survival after 6 months storage		Incubation temperature	Technique for viable count
	0*	6		
Group VII				
<i>L. brevis</i> X1	56.7	23.9	30°	(<i>a</i>)
<i>L. brevis</i> X2	71.0	68.0		
<i>L. pastorianus</i> T5	73.1	28.6		
<i>L. pentoaceticus</i> E2	67.2	18.2		
<i>L. buchneri</i> BC1	88.5	266.7		
<i>L. brevis</i> BR11‡	52.3	62.4		
<i>L. brevis</i> SL15‡	25.4	0.9		
Group VIII				
<i>L. fermenti</i> F1	72.9	80.6	37°	(<i>a</i>)
<i>L. fermenti</i> F4	43.9	28.0		
<i>L. fermenti</i> AH18B‡	46.5	48.5		
Section I				
<i>L. jugurt</i> J1	61.8	48.6	37°	(<i>b</i>)
<i>Lactobacillus</i> AH4‡	29.1	14.1		
Section II				
<i>Lactobacillus</i> RF1‡	79.2	42.5	37°	(<i>a</i>)
Section III				
<i>L. acidophilus</i> A7	123.9	112.8	37°	(<i>a</i>)
<i>L. casei</i> C28	96.5	65.0		
Section IV				
<i>L. bifidus</i> S6	10.9	0.4	45°	(<i>b</i>)

* Immediately after freeze-drying.

† Result of duplicate experiments.

‡ Freshly isolated strains.

Plating techniques (see p. 504): (a) double layer agar plates; (b) single layer plates in anaerobic jars.

Since, in addition to errors in technique, particularly in dilution and plating, the apparatus used might have been responsible for some variations, comparisons were made between this and an Edwards's (W. Edwards and Co. (London) Ltd.) model L5 (centrifugal) freeze-drier. However, there was little difference between the results obtained with the two sets of apparatus, variability being equally great and survival rates similar.

Our observations agree closely with those of Fry & Greaves (1951); they emphasize the importance of determining viable numbers and percentage survivals after freeze-drying and during storage. They also recognize the importance of the suspending medium and advise the inclusion of 5 to 10 % of lactose or glucose. No significant differences were found in their results when the number of organisms to be dried was varied, but an 'old' culture (20 hr.) dried more satisfactorily than a young one (4 hr.). (The youngest cultures of lactobacilli dried were 24 hr. old.) Fry & Greaves suggested that their methods

and results might be applicable to other species of bacteria, and aimed at a survival of 1 % over several years; it now seems that this should not be difficult to attain with the lactobacilli. The figures in Table 6 justify, from the

Table 6. *Distribution of survival rates from Table 5 in the range 0 to 100 + %*

Arbitrary divisions of survival rate range 0 to 100 + %	Number of results in each arbitrary division	
	Immediately after freeze-drying	After 6 months storage
0 to 1 %	0	5
1 to 10 %	6	8
10 to 25 %	6	11
25 to 50 %	11	13
50 to 100 + %	29	15

standpoint of survival rate, the methods used for freeze-drying lactobacilli. Physiological and serological studies of lactobacillus cultures and antisera freeze-dried in this work are described by Sharpe & Wheeler (1955).

We wish to thank Dr A. T. R. Mattick for helpful advice and suggestions.

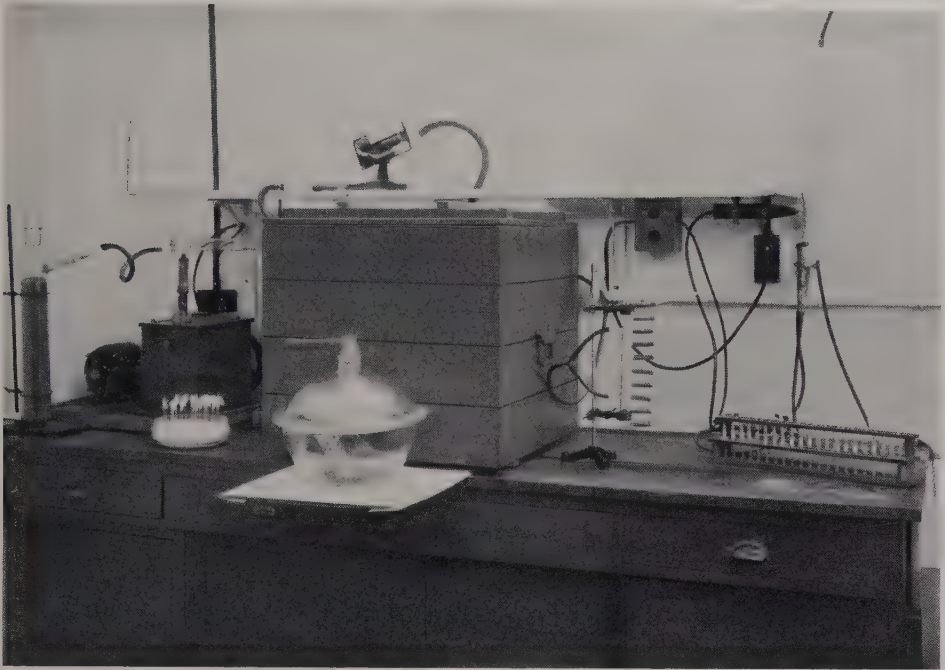
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EXPLANATION OF PLATE

Freeze-drying apparatus showing silica gel column, vacuum pump, freeze-drying tubes in dish of powdered solid CO₂, 'Vacustat', insulated box, desiccator containing basket of drying tubes and dish of phosphorus pentoxide, manifold, vacuum tester, jeweller's flame and rack of completed tubes ready for storage.

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M. BRIGGS, G. TULL, L. G. M. NEWLAND & C. A. E. BRIGGS—FREEZE-DRYING OF
LACTOBACILLI. PLATE 1

(Facing p. 512)

SHARPE, M. E. & WHEATER, D. M. (1955). *J. gen. Microbiol.* 12, 513-518

The Physiological and Serological Characters of Freeze-dried Lactobacilli

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SUMMARY: A comparison of physiological and serological characters of 41 strains of lactobacilli before freeze-drying and after freeze-drying and storage for 6 months showed that freeze-drying did not materially alter these characters.

If there were some degree of selection, or the loss of some enzyme, antigen or other characteristic of organisms during the process of freeze-drying, strains with properties differing from the original would result. Before freeze-drying a collection of strains of lactobacilli (Briggs, Tull, Newland & Briggs, 1955) it was therefore necessary to determine whether freeze-drying did in fact alter the physiological and serological characters of the organisms dried. Elser, Thomas & Steffen (1935) freeze-dried strains of meningococci and gonococci and found them to be unaffected, and Cowan (1951, 1954) found that cultures examined by biochemical tests before and after they were dried showed no significant differences. Several authors have also reported the retention of serological properties (Stamp, 1947; Flosdorf, 1949; Proom & Hemmons, 1949; Jennens, 1954).

The physiological and serological properties of the strains of lactobacilli used for the present work had been examined before freeze-drying by Briggs (1953), Wheeler (1955*a, b*) and Sharpe (1955). This paper reports a comparison of the results of these tests on organisms before freeze-drying and after freeze-drying and storage for 6 months. An account of the results of freeze-drying 12 lactobacillus antisera is also included.

METHODS

Strains. The 41 strains which were tested were representative of all groups and sections described by Briggs (1953). Cultures were dried by Briggs *et al.* (1955), who also tested the rates of survival. Tubes were opened after 6 months storage and subcultured into tomato glucose broth (Briggs, 1953). Four further daily sub-cultures were made and strains were then tested by the methods given below.

Physiological tests

The media and methods used for the following tests were those of Briggs (1953): production of gas from glucose, gas from citrate and ammonia from arginine; heat survival; growth temperatures; tolerance of NaCl. Carbohydrate fermentation tests (Wheeler, 1955*a*) were done on a few of the strains. Where

the result obtained differed from those shown by the original strain, the original strain which had been kept in yeast glucose litmus milk + chalk at 4° (Briggs, 1953) was retested.

Serological tests

Precipitin tests. HCl extracts of the strains were tested by ring precipitin tests against grouping sera for lactobacilli and also against homologous type sera when available (Sharpe, 1955).

Agglutination tests. Washed standard suspensions were tested by tube-agglutination tests against appropriate sera. For comparison, tests were put up concurrently with the original strains which had not been freeze-dried.

When any strains showed different precipitin or agglutination reactions, fresh tubes of freeze-dried cultures were used for repeat tests.

RESULTS

Physiological tests

Table 1 shows the results of these tests.

Gas from glucose and ammonia from arginine. With one exception in the arginine test the results of the tests on freeze-dried cultures were identical with those given by the organisms before freeze-drying.

Gas from citrate. Four strains gave different reactions to this test after freeze-drying.

Growth temperatures. Of the 41 strains tested, 39 showed the same results at 15°, 45° and 48° as the original strains. One strain failed to grow at 15° after drying, and another grew at 15° after drying, but not before.

Heat survivals. Fifteen freeze-dried strains survived heating for a longer, and two for a shorter, time than the original strains; the remainder gave the same results before and after freeze-drying.

Tolerance of 4, 6 and 8 % NaCl. Ten strains grew in a higher concentration of salt after freeze-drying than before; 2 strains did not grow in as high a concentration as before drying.

Carbohydrate fermentations. Only two strains of *Lactobacillus acidophilus* and two of *L. bulgaricus* were tested; none showed a change in fermentation reactions after drying.

Serological tests

Precipitin reactions. Thirty-seven strains showed no change in their precipitin reactions; one strain of *L. acidophilus* gave a weaker type reaction than the original, but the group reaction was unaltered; three strains did not react with any available sera either before or after freeze-drying.

Agglutination reactions. Table 2 shows that 21 freeze-dried strains reacted to the same titre with the sera as the original strains; 11 strains gave a reaction either one tube higher or one tube lower than the original ones; 4 strains did not react with any of the available sera either before or after freeze-drying; 1 strain showed auto-agglutination with suspensions of both cultures; 2 freeze-dried strains showed auto-agglutination, whereas smooth suspensions were

Table 1. Differences in physiological characters of lactobacilli after freeze-drying

Strain	Gas from glucose	Gas from citrate	NH ₃ from arginine	Heat survivals					Growth at			Tolerance to NaCl		
				At 60° for		At 65° for		15°	45°	48°	4 %	6 %	8 %	
				30 min.	60 min.	90 min.	90 min.							
<i>L. acidophilus</i> A 4	.	.	.	—	.	+
<i>L. acidophilus</i> BF 4	.	.	.	+	+	+
<i>L. bulgaricus</i> Y 48	+
<i>L. lactis</i> L 3	+
<i>L. lactis</i> A 6
<i>L. helveticus</i> C 10	.	.	.	+
<i>L. helveticus</i> YOG 3	.	+	.	+	+	+	+	.
<i>L. casei-helveticus</i> O 9	+	+	+	.
<i>L. casei</i> C 5	.	—	.	.	+	+	+	.
<i>L. casei</i> C 24	.	.	.	+	+	+
<i>L. casei</i> C 28	+
<i>L. plantarum</i> P 4	+
<i>L. plantarum</i> SL 5	.	.	+	+
<i>L. fermenti</i> F 1	.	—	—	+
<i>L. fermenti</i> F 4	+	.	.
<i>L. fermenti</i> PF 3	.	.	.	+	+	+
<i>L. fermenti</i> AH 18 B	+	.	.
<i>L. leichmannii</i> LE 6	+	.	.
<i>L. buchneri</i> BC 1	.	.	.	+	+	.	.
<i>L. pastorianus</i> T 5	+
<i>Lactobacillus</i> sp. A 7	—	.
<i>Lactobacillus</i> sp. RF 1	+	+
<i>Lactobacillus</i> sp. AH 4	+	+
<i>Lactobacillus</i> sp. PR 8	.	.	.	+	+	+

The reactions of 5 strains of *L. acidophilus*, 3 strains of *L. bulgaricus*, 3 strains of *L. brevis*, 2 strains of *L. lactis*, 1 strain of *L. leichmannii*, 1 strain of *L. thermophilus* and 2 unclassified strains remained unaltered after freeze-drying.
 + = reaction negative before freeze-drying, positive after freeze-drying. — = reaction positive before freeze-drying, negative after freeze-drying.
 . = reaction remained unaltered.

obtained from the original strains. Fresh tubes of the 2 freeze-dried strains which showed auto-agglutination were subcultured daily into yeast glucose litmus milk for 5 days before being transferred into tomato glucose broth. After this treatment auto-agglutination of the two strains was considerably diminished.

Two strains of *Lactobacillus fermenti* gave anomalous reactions even after repeated testing. With one strain (F1) the agglutination titre after drying was much decreased; with the other strain (AH18B) no agglutination reaction was obtained before drying but it agglutinated to a high titre after drying. HCl extracts of both strains gave equally strong precipitin reactions before and after drying.

Although the agglutination titres of the freeze-dried and original strains were so similar, it was observed that there was some auto-agglutination with several of the suspensions made from freeze-dried strains. This did not happen with the original strains, suggesting an increased sensitivity to salt.

Table 2. *Differences in agglutination reactions of lactobacilli after freeze-drying*

Strain	Agglutination titres against homologous type sera	
	Before drying	After drying
<i>L. acidophilus</i> BF4	640	1280
<i>L. bulgaricus</i> Y48	80	160
<i>L. leichmannii</i> LE6	2560	5120
<i>L. lactis</i> AH7	320	640
<i>L. buchneri</i> BC1	320	640
<i>Lactobacillus</i> sp. AH4	320	640
<i>Lactobacillus</i> sp. CS7	160	320
<i>Lactobacillus</i> sp. J1	640	1280
<i>L. bulgaricus</i> B2	1280	640
<i>L. casei</i> C28	80	40
<i>L. casei-helveticus</i> O9	5180	2560
<i>L. fermenti</i> F1	2560	160
		(very slight agglutination)
<i>L. fermenti</i> AH18B	No reaction	2560
<i>Lactobacillus</i> sp. RF1	40	Auto-agglutination
<i>L. brevis</i> SL15	20	Auto-agglutination
<i>L. brevis</i> X2		Auto-agglutination

The agglutination reactions of 5 strains of *L. acidophilus*, 3 strains of *L. lactis*, 2 strains each of *L. bulgaricus*, *L. casei*, *L. helveticus*, *L. fermenti* and *L. brevis*, 1 strain each of *L. leichmannii*, *L. plantarum* and *L. thermophilus* were the same after drying as before. No sera were available for testing the 4 remaining strains.

Freeze-dried antisera

Immediately after freeze-drying (Briggs *et al.* 1955) and 3-6 months later, twelve freeze-dried lactobacillus antisera were reconstituted and tested by precipitin ring tests against HCl extracts of lactobacilli. Ten sera were found to have retained their potency and specificity; with one of these sera the potency after 6 months was markedly higher than that of the same serum

stored in the liquid form, which had deteriorated. The two other sera showed on reconstitution marked opalescence, which could not be removed by centrifuging and which rendered them unsuitable for precipitin tests. Elser *et al.* (1935) and Flosdorf (1949) mention the appearance of turbidity in some of their freeze-dried sera on reconstitution, because of lyophobic lipoidal constituents of serum; they overcame the difficulty by double processing with intermediate Seitz filtration.

DISCUSSION

In most cases the physiological reactions were unchanged by freeze-drying. Where a change occurred, it was one of degree rather than entire loss or gain of a distinguishing characteristic. One strain, isolated from pig faeces, produced gas from glucose and ammonia from arginine after freeze-drying, both tests having been negative before. When the original strain was retested, it was found that these tests were now positive when a larger inoculum of an actively growing culture was used. (It was later confirmed serologically that this was a strain of *Lactobacillus fermenti*.) Similarly, three strains of *L. bulgaricus* gave growth temperatures and heat survivals typical of that species after but not before freeze-drying, and again retests of the original cultures when they were growing well agreed with those of the freeze-dried strains. It has been noticed that many strains of lactobacilli give better growth after freeze-drying than after storage in yeast glucose litmus milk, and differences noted above were due to poor growth of the strains in the original tests and not to the effect of freeze-drying; actively growing cultures and a good medium are essential for these tests (Briggs, 1953).

Some slight degree of selection favouring the more resistant cells occurs during the process of freeze-drying. With nearly half the strains there was a slight increase in tolerance of NaCl or in the heat resistance of the organisms. It was never sufficient to require reclassification of a strain into a different group, but the results of these physical tests show that they must always be used with caution. It has been found that the vitamin B₁₂ requirement of *Lactobacillus leichmannii* LE2 was unaltered after freeze-drying and storage for a month (Dr M. E. Gregory, personal communication), which is important in view of the widespread use of these organisms in microbiological assays.

The serological characteristics of the lactobacilli examined were also found to be unaltered by freeze-drying, except that the precipitin type reaction of one freeze-dried strain was somewhat weaker than that of the original strain and two strains gave anomalous agglutination reactions. It is of interest that the culture which gave a weaker precipitin reaction still agglutinated to full titre, and the two cultures which gave irregular agglutination reactions gave identical precipitin reactions before and after drying, indicating, as Sharpe (1955) has already observed, the possibility of more than one type antigen, the presence of which is indicated only by the appropriate test.

The increased sensitivity to salt observed in agglutination suspensions with several of the freeze-dried organisms was also observed by Cowan (1951) with some of his strains when first recovered from the dried state. He suggested

that this may have been due to agglutination caused by naturally occurring antibodies in the horse serum used for drying the cultures. As lactobacillus antibodies were not detected in any of the batches of horse sera tested for them, such a possibility is unlikely with our strains.

The results of the present work indicate that the lactobacilli tested retained their physiological and serological characters on freeze-drying.

We wish to thank Dr A. T. R. Mattick for his interest in this work, and Miss P. Burrows for technical assistance.

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CAYTON, H. R. & PRESTON, N. W. (1955). *J. gen. Microbiol.* **12**, 519-525

Spirillum mancuniense n.sp.

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SUMMARY: A new species of *Spirillum* has been isolated from grass cuttings. Main characters: vacuoles and volutin granules; motile by lophotrichous or amphitrichous flagella; no spores; Gram-negative; good growth in 0.5 % Evans peptone; no growth in meat extract or grass infusion; inhibited by 0.5 % (w/v) sodium chloride; catalase-negative; microaerophilic; good growth at 4°; optimum temperature 28°. The species has been designated *Spirillum mancuniense*.

An infusion of grass cuttings from a compost heap was found to contain a short spirillum. Although the organism was grossly out-numbered by other organisms, it was particularly striking on account of its active motility which was maintained for several days. The experimental results recorded here are the outcome of an attempt to isolate this organism in pure culture.

The isolation of the organism

Initial isolation in pure culture. The first step in the isolation of the spirillum in pure culture was the search for a solid medium on which it would grow. As nutrient agar was unsuccessful an attempt was made to simulate the cultural conditions under which the organism had already grown. A medium was prepared consisting of an autoclaved mixture of the compost infusion described above, hay infusion, and water from a bucket which had been standing out-of-doors for several weeks, the whole being solidified with 2 % (w/v) agar. After incubation for 2 days at 23°, a stained film of the confluent growth revealed a sufficiently large proportion of spirilla to suggest that multiplication of this organism had occurred; but films made from numerous isolated colonies consisted only of cocci, bacilli and fungi. However, by examining the culture under the 16 and 4 mm. objectives of a microscope, growth of the spirillum could be detected. (Pl. 1, figs. 1 and 2, shows the microscopic appearance of spirillum colonies in pure culture as finally obtained.) Prolonged search of several plates revealed a spirillum colony which was sufficiently isolated (Pl. 1, fig. 3) to enable a pure subculture to be made.

Repeated subculture in reproducible medium. The initial isolation in pure culture from a colony was made in filtered autoclaved water from a bucket which had been standing out-of-doors for several weeks, this medium being used quite empirically for no better reason than its previous use as a component of a successful solid medium. However, it obviously lacked reproducibility, and moreover the growth gradually became less and less profuse and eventually ceased after 43 subcultures, even when large inocula were used. Meanwhile

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various combinations of Yeastrel, hay infusion and grass infusion were tried. These were either unable to support growth at all, or were adequate for not more than three subcultures. The organism would not grow in standard laboratory media such as nutrient broth, digest broth, glucose broth, peptone water (containing sodium chloride), or Koser's citrate. It still thrived, however, after 100 subcultures in a solution of 0.5% (w/v) peptone (Evans) in distilled water, the reaction being adjusted to pH 7.5 before sterilization in the autoclave. Oxoid peptone gave equally good growth, as indicated by three subcultures, but cultures in Difco Bactopeptone and Witte peptone were less turbid.

Description of the organism

Morphology. The size of the organism in 0.5% Evans peptone was as follows: diameter $0.7\text{--}1.0\mu$. (most were about 0.9μ .), wavelength (λ) $7\text{--}8\mu$., spiral width $2\text{--}3\mu$. The average length was about $7\text{--}8\mu$. ($=\lambda$); many were short vibrio forms ($\lambda/4$), but lengths of 2λ and 3λ were common, and occasionally the spirillum reached a length of $30\text{--}40\lambda$ or more (Pl. 2, figs. 4, 5). Most of the organisms in young cultures stained uniformly with simple stains such as methylene blue (Pl. 2, fig. 6), but as the cultures aged some of the organisms went through a series of changes in their internal structure. After incubation for 1 day at 28° , some organisms developed a series of vacuoles along their length. Pl. 2, fig. 7, shows an unusually high proportion of organisms of this type. After 10 days, very few vacuoles were left, but many organisms had developed a number of granules with a distribution similar to that of the vacuoles seen in younger cultures (Pl. 2, fig. 8). Both the granules and the few remaining vacuoles were beginning to cause slight bulging of the spirillum. By the 24th day, many organisms had been grossly distorted by swollen granules, many of which had coalesced to form short fat curved bodies which stained intensely (Pl. 2, fig. 9). Some of these swollen granules had become almost globular (Pl. 2, fig. 10); occasional organisms contained unstained vacuoles of a similar size. Pl. 2, fig. 11, shows two organisms which contain large pale structures intermediate between the unstained vacuoles and the deeply stained granules. There was little change in the appearance of cultures during the next few weeks, but by the 15th week the organisms consisted of short spiral forms (which had been present at all stages) and ill-defined poorly staining globular forms (Pl. 2, fig. 12). No granules were seen at this stage.

Electron microscopy. Suspensions of young cultures grown on 0.5% Evans peptone solidified with 2% (w/v) Oxoid agar (New Zealand, L10) were examined by the electron microscope, using electron-accelerating voltages of 25 and 75 kV. The former voltage revealed tufts of flagella at one or both poles (Pl. 2, fig. 13) whilst the internal structure was poorly differentiated. At 75 kV. the flagella were less distinct, but the cytoplasm was seen to contain well-defined dense bodies and also some ill-defined areas which had but little deflecting effect on this faster electron beam (Pl. 2, fig. 14). The relation between these internal structures and the vacuoles and granules seen in stained films is discussed later.

Staining. The best contrast between granules and cytoplasm was obtained by staining heat-fixed films with Mayer's haemalum for 5 min. With this method, the granules appeared as sharply demarcated violet areas in a pale purple cytoplasm. Polychrome methylene blue stained the granules purple and the cytoplasm blue, but the intensities of the two colours were more nearly equal and, in consequence, the contrast between granules and cytoplasm was less marked. With Neisser's method, the granules stained black and the cytoplasm brownish yellow, but the granules absorbed so much stain that they lacked definition. Neither the granules nor the vacuoles gave positive staining reactions for spores (Moeller's method), fat (Sudan IV, and Sudan Black B), or sulphur (sodium nitroprusside). Films made from overnight cultures stained uniformly Gram-negative.

Cultural characters. The only medium in which the organism was found to grow profusely in repeated subculture was 0.5 % Evans peptone. Sodium chloride was inhibitory even in low concentrations (Table 1). Other anions and cations showed similar or more marked degrees of inhibition (Table 2).

Table 1. *Growth of spirillum in presence of sodium chloride, using 0.5 % Evans peptone as basal medium*

Concentration of sodium chloride % (w/v)	Growth	Motility
0	+++	Active
0.2	+++	Active
0.3	++	Active
0.4	+	Reduced
0.5	±	Very sluggish
0.6	—	—

Nutrient broth did not support growth; omission of sodium chloride from this medium gave no improvement. A simple meat extract (i.e. nutrient broth from which both salt and peptone had been omitted) gave a slight growth on primary subculture but subsequent subculture in the same medium was unsuccessful. On potato, the growth was very scanty and greyish in colour. Gelatin (5 %, w/v) stab cultures liquefied very slowly, the maximum growth occurring about 5 mm. below the surface. On a medium consisting of 0.5 % Evans peptone solidified with 10 % (w/v) gelatin, the colonies were small (up to 1 mm. in diam.) and greyish in colour.

Good growth was obtained on 0.5 % Evans peptone solidified with 2 % (w/v) Oxoid agar (New Zealand, L10), the colonies reaching a size of 2–3 mm. diam. when grown aerobically for 3 days at 28°; under strictly anaerobic conditions the growth on this medium was less profuse. But a diminution of air pressure to 60 mm. Hg resulted in slightly better growth than that obtained at atmospheric pressure. This preference for microaerophilic conditions was confirmed by the growth in gelatin stab cultures described above.

Temperature range. Liquid cultures were markedly turbid after overnight incubation at 28°, which was the optimum temperature. Below and above this temperature growth was less profuse, but the organism grew slowly even at 4°

At temperatures above 28° the growth rate declined more rapidly than below 28°; at 36° there was only a trace of growth and the organisms were non-motile. No growth occurred at 44°, and after 2 days at this temperature the organisms were dead.

The absence of heat-resistant spores, suggested by the above result, was confirmed by heating 10-day, 4-week, and 12-week cultures at 60° for 30 min. All three cultures were killed. The vegetative form of the organism, however, could itself survive for several weeks at room temperature; cultures were still viable after 10 weeks but not after 15 weeks.

Table 2. *Relative growth of spirillum in presence of equivalent concentrations of various anions and cations, using 0.5 % Evans peptone as basal medium*

Salt	Growth in higher salt concentration						Growth in lower salt concentration					
	Concentration of salt	Period of incubation (days)					Concentration of salt	Period of incubation (days)				
		1	2	3	5	8		1	2	3	5	8
NH ₄ Cl	M/10	—	—	—	±	+	M/20	++	++	++	++	++
KCl	M/10	—	—	—	±	+	M/20	++	++	++	++	++
NaCl	M/10	—	—	—	—	+	M/20	++	++	++	++	++
CaCl ₂	M/20	—	—	—	±	+	M/40	—	+	++	++	++
MgCl ₂	M/20	—	—	—	—	—	M/40	—	—	—	—	—
FeCl ₃	M/30	—	—	—	—	—	M/60	—	—	—	—	—
LiCl	M/10	—	—	—	—	—	M/20	—	—	—	—	—
(NH ₄) ₂ SO ₄	M/20	—	—	±	+	++	M/40	++	++	++	++	++
K ₂ SO ₄	M/20	—	—	—	±	+	M/40	++	++	++	++	++
Na ₂ SO ₄	M/20	—	—	—	—	—	M/40	+	+	+	+	+
MgSO ₄	M/20	—	—	—	—	—	M/40	—	—	—	—	—
FeSO ₄	M/20	—	—	—	—	—	M/40	—	—	—	—	—
MnSO ₄	M/20	—	—	—	—	—	M/40	—	—	—	—	—
KNO ₃	M/10	—	—	—	—	—	M/20	—	+	++	++	++
NaNO ₃	M/10	—	—	—	—	—	M/20	—	—	—	±	+
Na ₂ CO ₃	M/20	—	—	—	—	—	M/40	—	—	—	—	—
NaF	M/10	—	—	—	—	—	M/20	—	—	—	—	—
Na citrate	M/30	—	—	—	—	—	M/60	—	—	—	—	—

++, +, ± = degrees of growth; — = no growth.

Biochemical reactions. Using 0.5 % Evans peptone as the basal medium, it was found that the spirillum did not ferment glucose (22 days at 30°), did not produce indole, and did not reduce nitrate to nitrite. Catalase was not produced by growth on peptone agar.

Preservation. It was not possible to preserve the spirillum by freeze-drying in serum, and only a low survival rate was obtained with 7.5 % (w/v) glucose as the suspending fluid. But more successful results were obtained with the *Mist. desiccans* of Fry (1951) using 0.5 % Evans peptone in place of nutrient broth. By this method, viable organisms were recovered from all ampoules opened within a week of freeze-drying *in vacuo*, but after storage for 4 months at room temperature there were no survivors.

The organism could be maintained by monthly subculture in 0.5 % Evans peptone at 4°; this method was completely reliable.

Pathogenicity. The spirillum was non-pathogenic to mice and guinea-pigs. These animals were still healthy 2 months after intraperitoneal injection with 0.5 ml. of a heavy suspension of the organism.

DISCUSSION

The classification of this organism does not present any great difficulties. Its general characters (spiral shape, presence of volutin granules, tufts of polar flagella, origin in decaying organic matter) place it in the genus *Spirillum* as described in *Bergey's Manual* (1948). It belongs to the subgroup of smaller organisms which develop volutin granules, but differs from previously described spirilla of similar size in several of its properties, the most striking of which are its inability to grow in broth, its very poor growth on potato, and the absence of catalase. These differences seem to justify species differentiation.

Although species of similar size to this new strain have been grown on several artificial media, some other spirilla have not been so cultivated. The organism described in this communication has grown well in repeated subcultures only in a simple solution of Evans peptone to which no salt was added. In fact, one of the peculiar features of this strain is its inhibition by sodium chloride in a concentration as low as 0.5 % (w/v). Possibly this inhibition may apply more widely and may explain the recorded failures of other species to grow in artificial media. In this connexion the work of Kutscher (1895) and Giesberger (1936) is relevant. *Spirillum kutscheri* grew in 'Fleischwasser' but not in a peptone common salt solution (salt concentration not stated). Giesberger, likewise, did not define the 'Peptonwasser' and 'Peptonagar' which supported growth; the text implies the absence of added salt but there is nothing to show that common salt is inhibitory.

The literature is lacking in detailed morphological descriptions of spirilla, so that the interesting changes found in ageing cultures of this new species might apply to other species. Rows of vacuoles in very young cultures are mostly replaced, by the end of a week, with similar rows of volutin granules which subsequently increase enormously in size so that they grossly distort the former spiral shape of the cells. Occasionally, swollen vacuoles of a similar shape are seen; Pl. 2, fig. 11, shows two such vacuoles staining faintly with the same reaction as the more common swollen granules. These findings suggest that the granules develop from the vacuoles as the latter become filled with volutin material. No function has been ascribed to these swollen and globular forms of the organism, and they have not been shown to play any part in a 'life-cycle' of the spirillum. It would indeed be interesting to isolate cells of this type and determine whether they were capable of reversion to the original spiral shape. At present, however, they appear to be merely degeneration forms.

Electron micrographs of young cultures show small opaque bodies which occur in areas quite separate from the vacuoles. This may seem to refute the theory that vacuoles develop into volutin granules, but on the other hand there is no reason to suppose that an area which has a marked deflecting effect

on a beam of electrons is necessarily the same as one which stains as a volutin granule. Further light may be thrown on this problem by electronmicrography of older cultures and comparison with stained preparations.

Although this organism has several properties not previously described amongst species of *Spirillum*, further investigations may reveal that one or more of these characters is common to other species. Moreover, some of these newly described properties may well be shared with species which have not yet been isolated. It therefore seems to be safer, in providing a name for the organism, to avoid a term which describes an individual morphological or physiological character. Accordingly, the new species has been designated *Spirillum mancuniense*.

Main features of the new species

Spirillum mancuniense n.sp. Spiral organism 0.7–1.0 μ . in diam., wavelength 7–8 μ ., spiral width 2–3 μ ., length 2–300 μ . Motile by tufts of polar flagella. Vacuoles and volutin granules. No spores, fat, or sulphur. Gram negative. Colonies on peptone-agar smooth, round, greyish, 2–3 mm. diam.

Microaerophilic, facultative aerobe and anaerobe. Optimum temperature 28°; good growth at 4°. Vigorous growth in 0.5 % (w/v) Evans peptone. Very scanty growth on potato; no growth in meat extract or nutrient broth. Inhibited by 0.5 % (w/v) sodium chloride. Slow liquefaction of gelatin. No fermentation of glucose. Indole not produced. Nitrate not reduced. Catalase negative. Non-pathogenic. Isolated at Manchester from decaying grass cuttings.

A subculture has been deposited in the National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London, N.W. 9 (NCTC no. 9582).

The authors are indebted to Messrs Metropolitan-Vickers Ltd. for the electron micrographs.

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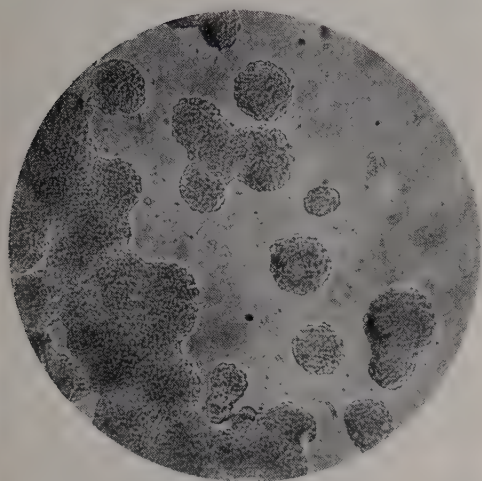
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EXPLANATION OF PLATES

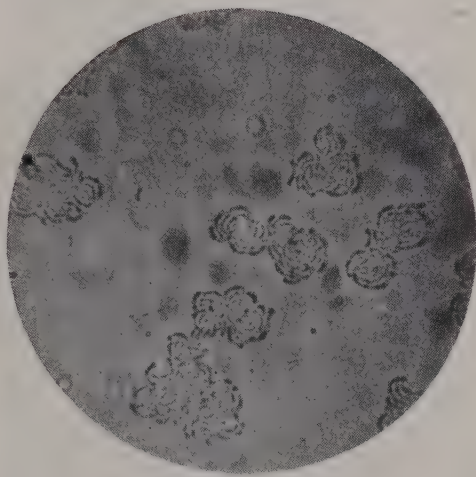
PLATE 1

Photomicrographs of colonies of *Spirillum mancuniense*

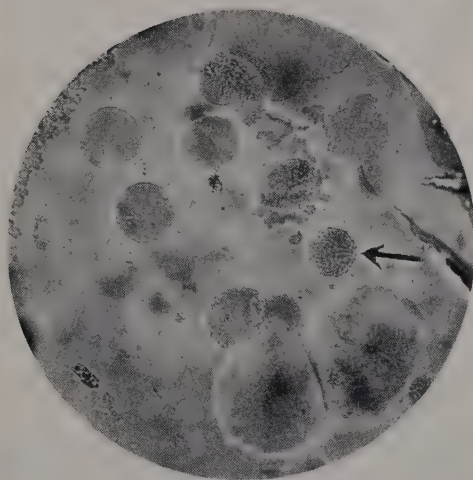
- Fig. 1. Pure cultures after 2 days at 23°. Colonies have undulating edge. $\times 200$.
- Fig. 2. Same culture as fig. 1, after 1 day at 23°. Shows how the spiral organisms produce the undulating edge seen with lower magnification in fig. 1. $\times 700$.
- Fig. 3. Mixed culture showing one isolated colony of spirillum (arrowed). $\times 200$.



1



2



3

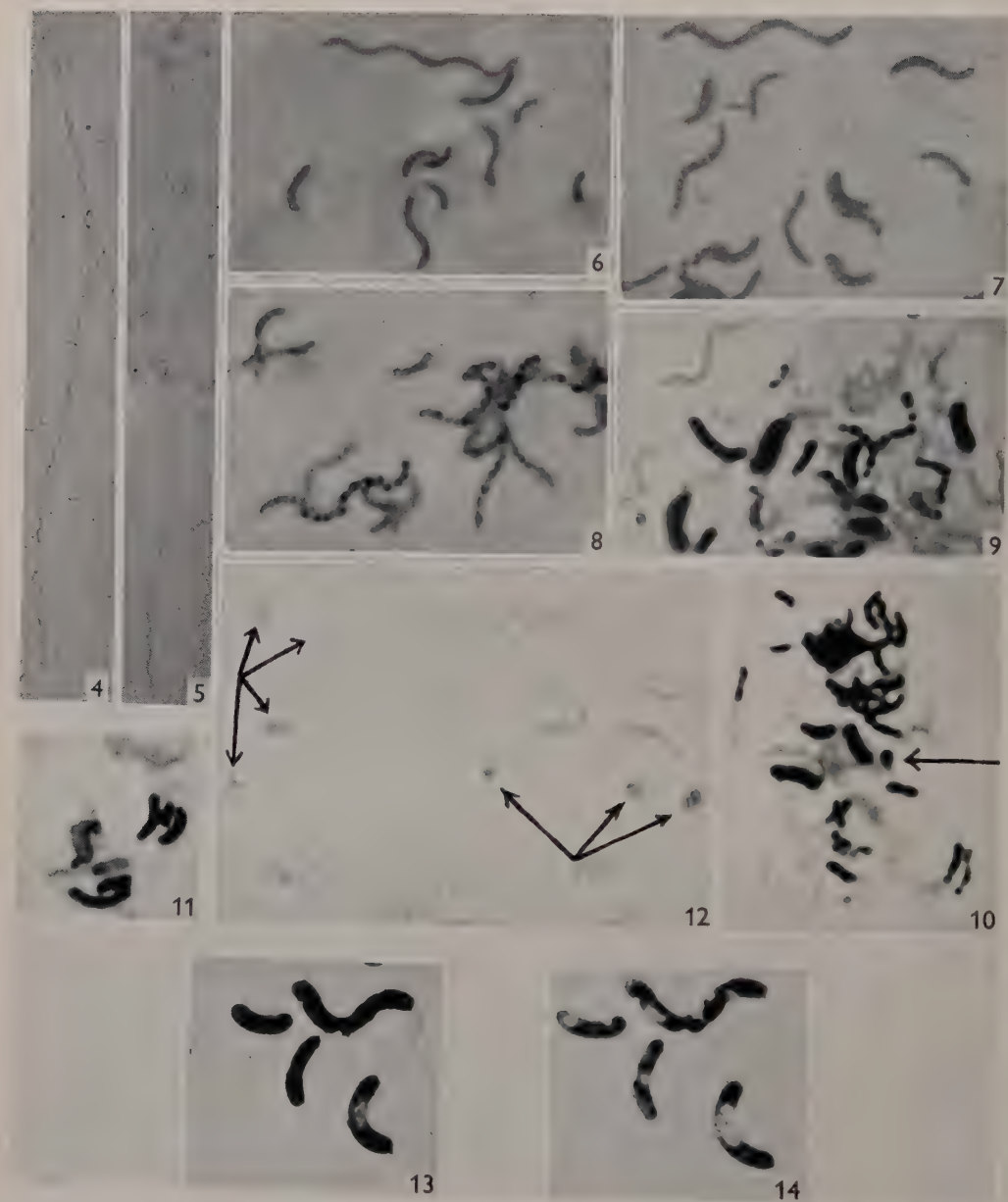


PLATE 2

Photomicrographs of *Spirillum manconiense*, stained with haemalum,
after growth in 0.5 % (w/v) Evans peptone at 28°

- Fig. 4. An organism of about 30 wavelengths in a 2-day culture. $\times 375$.
Fig. 5. A similar spirillum seen in a 3-week culture. $\times 450$.
Fig. 6. A typical field, showing uniform staining of cells in a young culture (3-day). $\times 1200$.
Fig. 7. One-day culture. This field shows an unusually high proportion of cells with vacuoles. $\times 1350$.
Fig. 8. Ten-day culture. Vacuoles mostly replaced by volutin granules. $\times 1200$.
Fig. 9. Twenty-four-day culture. Cells becoming distorted as the granules swell and coalesce. $\times 1200$.
Fig. 10. Same as fig. 9. The arrow points to the final globular shape which the swollen granules assume. $\times 1200$.
Fig. 11. Same as fig. 9. Note the two cells with large pale granules, probably swollen vacuoles filling with volutin material. $\times 1200$.
Fig. 12. Fifteen-week culture. The globular granules have degenerated and are now ill-defined. Spiral forms of the organism are present in this and all younger cultures. $\times 1200$.

Electron micrographs of suspension of *Spirillum manconiense* from 3-day growth
on 0.5 % peptone solidified with agar.

- Fig. 13. Electron-accelerating voltage of 25 kV. Tufts of polar flagella are seen, but the internal structure of the cells is ill-defined. $\times 2500$.
Fig. 14. Electron-accelerating voltage of 75 kV. Internal structure is revealed better, but flagella are indistinct. $\times 2500$.

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The Isolation and Classification of Proteolytic Bacteria from the Rumen of the Sheep

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SUMMARY: Proteolytic bacteria were isolated from the rumen of three sheep receiving an adequate protein diet. Using selective anaerobic conditions to suppress the majority of bacteria which were not capable of utilizing casein, proteolytic representatives of several bacterial genera were found. All of these may have been derived from the environment or food of the sheep. The most frequent organism was *Bacillus licheniformis* which existed in the vegetative form in the rumen, although present mostly in the form of spores in the hay fed to the animal.

Since it was reported by Sym (1938) that the bovine rumen contained a highly active proteinase attributable to microbial origin, more recent observations by McDonald (1952), by Annison, Chalmers, Marshall & Synge (1954) and by Chalmers, Cuthbertson & Synge (1954*a, b*) have clearly shown that when protein (in the form of casein, gelatin, ground-nut meal, and to a less extent herring meal) is fed to sheep a rapid and extensive breakdown of the protein may occur. This was found to result in liberation of ammonia in the rumen, with a consequent loss in nutritive value of the protein to the animal. Since the organ itself elaborates no proteolytic enzymes this breakdown must be attributed to the micro-flora or micro-fauna of the rumen, either by the secretion of extracellular proteases, or by intracellular enzymes liberated into the rumen after death and autolysis amongst the microbial population. Liberated intracellular enzymes must be an inevitable component of such a complicated mixed culture as the rumen presents. But the presence of extracellular protease will be determined by the types of actively multiplying micro-organisms present.

Much work has been carried out recently upon the bacterial flora of the rumen, and many rumen bacteria have been isolated and studied in pure culture. Very few of these produced gelatinase. From the bovine rumen an anaerobic Gram-positive rod, designated RO-CL, was isolated by Huhtanen & Gall (1953*b*) which produced acetic and propionic acids from lactic acid, and which was proteolytic; Bryant & Burkey (1953*b*) described a Gram-positive gelatin liquefying rod which they designated +SR -gGXC.

In many cases the rumen bacteria which have been described were among the predominant types present and were isolated from high dilutions (Bryant & Burkey, 1953*a, b*); or they were selected by virtue of their cellulolytic (Hungate, 1950) or saccharolytic properties (MacPherson, 1953; Mann, Masson & Oxford, 1954; Mann & Oxford, 1954). Huhtanen, Rogers & Gall (1952) and Huhtanen & Gall (1953*a, b*) used what was in effect an enrichment method to cultivate bacteria of fastidious nutritional requirements by inoculation of high dilutions into broth. It seems evident that proteolytic bacteria,

if present in the rumen, are not usually amongst the predominant organisms, though they may nevertheless be of great importance in bringing about the first stage of protein degradation, thus providing substrates for attack by many other types of bacteria with subsequent breakdown of amino acids. It was considered therefore that a systematic investigation of the types of bacteria in the rumen of the sheep which produce extracellular protease *in vitro* might provide a useful addition to the existing knowledge of rumen microbiology.

METHODS

The method used for primary isolation of proteolytic bacteria was based upon that of Hungate (1950) but the medium was modified to have a selective effect upon the rumen population and to encourage the growth only of those bacteria capable of utilizing protein. To 45 ml. mineral salt solution 1 (0.3 % K_2HPO_4) and 45 ml. mineral salt solution 2 (0.3 % KH_2PO_4 , 0.6 % NaCl, 0.06 % $MgSO_4$, 0.06 % $CaCl_2$) was added 6 g. agar (Davis), 1 ml. resazurin solution (0.005 %), and either 1.5 g. yeast extract (Difco) or 90 ml. clarified rumen fluid, withdrawn from the rumen of a sheep upon a low protein diet. In some cases 1.5 g. peptone (Evans) was included. The volume was made up to 300 ml. with distilled water. This basal medium was boiled thoroughly and filtered through paper pulp; 200 ml. was then measured and sterilized by autoclaving. The following additions were made aseptically—34 ml. 6 % casein solution (B.D.H., light white soluble casein) previously sterilized by steaming, 13 ml. sterile 6 % Na_2CO_3 , 3.4 ml. sterile 3 % cysteine HCl. CO_2 was bubbled through the hot medium until the resazurin was reduced, and the pH value was varied from 6.8 to 7.4. This medium was transferred aseptically to sterile tubes (18 × 150 mm.) fitted with rubber bungs, under an atmosphere of CO_2 , in 8 ml. quantities, and kept at 48° until required.

Three sheep were used, all with rumen fistulas. Two of these, sheep 969 and sheep 70, were fed 300 g. ground-nut meal, 300 g. flaked maize and 300 g. hay daily. The third, sheep 245, received 100 g. hay with 400 g. stock diet (maize, oats, bran, linseed meal, white fish meal) daily. For each experiment rumen samples of about 30 ml. were withdrawn from deep within the rumen, and as short a time as possible was allowed to lapse between the collection of the sample and inoculation of the medium. Some samples were homogenized for 3 min. in a Waring blender; all were very lightly centrifuged. Serial 1/10 dilutions were prepared either in the anaerobic diluting fluid advocated by Bryant & Burkey (1953*a*) or in normal saline; 1 ml. volumes of appropriate dilutions were used as inocula for the previously prepared tubes of medium, in duplicate or triplicate, from which roll tube cultures were made. These were incubated at 38° for 4 or 5 days.

RESULTS

As this protein-containing medium is initially cloudy because of casein in suspension extracellular 'casease' could be clearly demonstrated in some cases by a zone of clearing around the colonies. This could not however always be

relied upon as a criterion for recognizing a caseolytic organism because in some cases the zone of clearing was so narrow as not to be readily visible in roll tube culture, even when on subculture protease could be demonstrated. On the other hand, with the less dilute inocula, a large area of medium cleared rapidly, and this extensive casein breakdown permitted active growth of colonies not in themselves proteolytic. All isolated colonies available were therefore transferred to slope cultures, purified, and inoculated into nutrient gelatin and litmus milk in the usual manner.

Total viable counts were similar for all three sheep, and, with an accurate technique, were of the order of 10^5 to 10^7 /ml. of rumen fluid, depending upon the composition of the medium. When peptone was included the $1/10^6$ dilution tubes were the most suitable for counting, and the predominant flora consisted of Gram-positive (usually catalase-negative) cocci, with smaller numbers of bacilli. With the addition of fermentable carbohydrate (glucose or cellobiose) the colony count was rather higher and the flora more mixed, but proteolytic anaerobes were not amongst the isolates. In the absence of peptone or carbohydrate, yeast extract as supplement allowed the development of a rather larger number of colonies than did rumen liquor, but with no gross difference in the predominant types developing; in either case bacilli and Gram-negative rods were most frequent, with smaller numbers of micrococci, corynebacteria, and Gram-negative diplococci. Sheep 245 differed from the other two in that many of the Gram-negative rods isolated produced acid and gas in lactose bile-salt broth; with sheep 70 and 969 coliform organisms were infrequent.

Of 176 cultures isolated 77 produced gelatinase and most of these 77 cultures also peptonized casein. All the proteolytic isolates were representative of bacterial genera likely to be found in the environment, food and water of the sheep. With the exception of clostridia, all were facultative anaerobes.

Bacillus species

The majority of proteolytic strains isolated at dilutions of $1/10^4$ to $1/10^7$ of rumen fluid were facultative anaerobic members of the genus *Bacillus*. Of 24 *Bacillus* isolates examined by the methods recommended by Gibson (1944) and by Smith, Gordon & Clark (1946) 19 isolates were clearly identified as *B. licheniformis*. Though these strains exhibited considerable differences in cultural form, from mucoid to rugose or adherent, with or without pigment, their biochemical properties showed them to be closely related. Morphology placed them indisputably in group 1 of Smith *et al.* (1946) with one exception—one strain only exhibited definite equatorial swelling of the mature sporangium, though in all other respects it was typically *B. licheniformis*. All strains liquefied gelatin and peptonized casein in litmus milk rapidly anaerobically at 38°. Fermentation of glucose, xylose and arabinose was, as found by Smith *et al.* (1946), shown more consistently on agar slopes containing an ammoniacal nitrogen source than on peptone agar, and only one strain failed to ferment arabinose, while two others did not ferment xylose. Other properties

agreed with those previously recorded by Knight & Proom (1950). These authors also have shown that *B. licheniformis* requires no growth factors, and all the rumen strains of this species grew well through several subcultures with an ammonium salt as the sole source of nitrogen. The Gibson-Abdel-Malek reaction was positive, though in some cases CO₂ production could be demonstrated convincingly only in 5 % glucose agar milk cultures, and not well in 5 % glucose soft gelatin cultures (Gibson & Abdel-Malek, 1945).

Two cultures appeared related to *Bacillus licheniformis*, but differed in not producing CO₂, and in being able to grow under decreased oxygen tension but not under strict anaerobic conditions. One culture, in which NO₃ reduction and diastase were not demonstrated, appeared to be *Bacillus pumilus*, and the second, by virtue of starch fermentation without NO₃ reduction, was probably a *subtilis-pumilus* intermediate.

Two cultures were identified as *Bacillus cereus*. The average width of these vegetative rods was 1-1.2 μ . Production of phospholipinase was shown by the egg-yolk reaction of McGaughey & Chu (1948). Xylose and arabinose were not fermented; glucose was fermented with formation of acetylmethylcarbinol, without CO₂, but contrary to the findings of Knight & Proom (1950) starch hydrolysis was not demonstrated. Gelatin and casein were attacked rapidly. These strains were not able to utilize ammoniacal nitrogen.

One culture of slender rods, Gram-positive only when very young, and sporulating infrequently within clavate sporangia, was identified as *Bacillus circulans*; both rough and mucoid variants were obtained. The organism liquefied gelatin and, unlike the majority of strains of *B. circulans* examined by Smith *et al.* (1946), peptonized casein in litmus milk. In all other properties observed the rumen strain agreed with the characters described by Smith *et al.* (1946) and by Knight & Proom (1950) for *B. circulans*.

All except two of the rumen bacilli examined grew well under anaerobic conditions at 38° upon an agar medium containing 40 % (v/v) rumen fluid from sheep 245 supplemented with 1 % casein, 0.1 % glucose, inorganic salts and bicarbonate buffer. Under these conditions endospores were very rarely seen, but vegetative growth took the form of pleomorphic Gram-positive rods and filaments, usually very granular. It was only when cultivated aerobically upon a suitable medium, and sometimes at a lower temperature, that the typical *Bacillus* morphology was exhibited. Similar granular pleomorphic rods which were perhaps bacilli were seen in Gram-stained films of rumen fluid.

That *Bacillus* spp. were in fact present in the rumen in the vegetative form rather than as endospores may be deduced from Table 1. After heating rumen fluid for 5 min. in a boiling water bath the numbers of organisms which could be isolated upon the casein roll tube medium were very significantly smaller than the numbers isolated in parallel counts without heating. To facilitate the recognition of bacillus colonies only, these counts were made in Petri dishes incubated under CO₂.

A search for *Bacillus licheniformis* in the hay fed to all three sheep showed the spores of this organism to be present in large numbers. Dry hay (10 g.) was thoroughly ground with 100 ml. broth, and the extract, unheated and after

heating in a boiling water bath for 5 min. plated anaerobically on nutrient agar. The spore count was of the order 10^5 /g. hay, and 15 colonies examined were all found to have the morphology of group 1 bacilli (Smith *et al.* 1946) and to give a positive Gibson-Abdel-Malek reaction, and were therefore presumed to be *B. licheniformis*. On plates of unheated hay extract bacilli were not found at a dilution greater than $1/10^6$, and it thus appears that the majority of facultative anaerobic bacilli in the hay were, in contrast to rumen fluid, in the form of spores.

Table 1. Colony counts of *Bacillus* spp. on casein medium in CO_2 with and without preliminary heating

Exp.	Sheep	Rumen fluid unheated. Dilutions			Rumen fluid heated. Dilutions		
		$1/10^3$	$1/10^4$	$1/10^5$	$1/10^1$	$1/10^2$	$1/10^3$
1	969	91	40	4	—	8 16	1 2
2	969	—	— c. 100	27 40	1 2	0 0	— —

Clostridium species

Four cultures of *Clostridium sporogenes* were isolated, from sheep 70 and 969. Because this actively proteolytic organism produces an alkaline reaction in peptone media, carbohydrate fermentation was not easy to detect by the usual method of inoculation into peptone water sugar broths and the results were variable. Much more consistent results were obtained on peptone agar slopes with the desired carbohydrates and indicator incorporated, as advocated by Smith *et al.* (1946) with reference to those *Bacillus* spp. which were not able to utilize inorganic nitrogen. Under these conditions acid was formed readily from glucose, fructose, galactose, sucrose or maltose, and after 5 days from salicin. Two strains in addition produced acid from xylose, raffinose, dulcitol and inulin after 5 days, while fermentation of starch, lactose and mannitol was consistently negative. Indole was not detected in peptone water cultures, but all other characters observed agreed with those described for *C. sporogenes* (Metchnikoff) in *Bergey's Manual* (1948).

Gram-negative rods

Of 45 isolates of Gram-negative rods only 5 liquefied gelatin, and none of these peptonized casein in litmus milk. Two pigmented strains appeared to be *Flavobacterium arborescens* (*Bergey's Manual*, 1948). Five non-pigmented strains were similar to each other; by virtue of their urease production, fermentation of glucose but not lactose, variable and uncertain production of gas in carbohydrate broth, and motility at 25° but not at 38° , they were considered to belong to the genus *Proteus* (*Bergey's Manual*, 1948); species names could not be assigned.

As Gram-negative rods in general quite frequently elaborate proteases, and as these organisms were evidently quite numerous in the rumen, it seemed

possible that the abundant proliferation of *Bacillus* spp. might mask the presence of proteolytic Gram-negative rods. The bacilli were therefore suppressed by the addition of 10 units penicillin/ml. to the roll tube cultures. The flora then became exclusively Gram-negative, and 19 rods were isolated; none of these liquefied gelatin.

Corynebacteria

Corynebacteria were isolated fairly frequently but only one culture, from sheep 70, was proteolytic. Morphology and method of cell division placed this bacterium clearly in the genus *Corynebacterium* but it was not possible to accord it a species name. Rods were Gram-positive, non-motile, and varied in size from coccoid (liquid media) to rods 3-4 μ . long. Growth on nutrient agar was abundant, aerobically and anaerobically, with formation of yellow pigment; in glucose broth a yellow viscous deposit was formed. When first isolated gelatin liquefaction was rapid; in litmus milk the reaction became alkaline but casein was not digested. Glucose, galactose, sucrose, lactose and maltose were fermented, but not xylose, dulcitol, mannitol, inulin or salicin. Orla-Jensen (1919) described *Microbacterium* spp. possessing properties like this organism in morphology, failure to ferment pentoses or alcohols, production of catalase, reduction of nitrates, and in surviving, in some numbers, a temperature of 80° for 2½ min. when heated in an agar medium.

Micrococci

Of several strains of Gram-positive catalase-positive cocci 6 produced partial liquefaction of glucose nutrient gelatin anaerobically within 7 days, without change of litmus milk. Their proteolytic activity did not in any way compare with that of the bacilli and clostridia. All cultures were non-pigmented; they all fermented glucose. Four strains were VP-positive and therefore belonged to subgroup 2 of Shaw, Stitt & Cowan (1951); 2 strains were VP-negative and therefore fell into their subgroup 3. Though the proteolytic powers were weak a fairly large range of carbohydrates was fermented, and urease was produced by all cultures.

The numbers of each group of organism isolated from individual sheep are recorded in Table 2.

Table 2. *Proteolytic isolates from rumen contents of hay- and concentrate-fed sheep*

Organism	Sheep numbers		
	70	969	245
	Isolates (no.)		
<i>Bacillus</i> spp.	19	24	18
<i>Clostridium</i> spp.	2	2	0
Gram-negative rods	4	0	1
<i>Corynebacteria</i>	1	0	0
Micrococci	4	2	0
% of isolates which were proteolytic	57	42	27

DISCUSSION

Isolations of proteolytic bacteria were made under such conditions of temperature, anaerobiosis, and bicarbonate buffer as would be expected to allow the development of 'true rumen bacteria' in the strict sense, i.e. species having their natural habitat in the rumen and nowhere else. If such organisms exist, there is undoubtedly also a large population of bacteria which are well known in other habitats, which are derived from external sources and are able to multiply within the rumen, and it is to this category that the proteolytic bacteria appear to belong. The most frequently isolated organisms were facultatively anaerobic *Bacillus* spp. These ubiquitous saprophytic bacteria are not commonly associated with an animal host, and although they grew well on a medium containing much rumen fluid, and under such physical conditions as are believed to obtain in the rumen, their metabolic activity there is difficult to assess. The numbers found bear a not insignificant relation to the total population, as the total viable count obtained upon a rich medium under the conditions used in these experiments was of the order 10^9 . The ratio of proteolytic to non-proteolytic bacteria was then, at the most, approximately 1 in 100. All the sheep used in these experiments were ingesting daily considerable numbers of bacilli from their feed in the form of spores, but spores were not detected to any great extent in the rumen, where vegetative forms were present in dilutions of $1/10^4$ to $1/10^7$. If, as appears to be the case, conditions within the rumen are suitable for the prompt germination of *Bacillus* spp. spores then it seems unlikely that multiplication would not follow. A parallel case may perhaps be found in the work of Gutierrez (1953) who reported the isolation of *Corynebacterium acnes* in considerable numbers from hay, and also from the rumen of cattle eating it, and this organism was believed to play an active part in the rumen propionic acid fermentation. The ration fed to the sheep of the present experiment was preponderantly carbohydrate. It would be interesting to determine whether a higher proportion of protein in the ration would result in a greater relative count of proteolytic bacteria.

I wish to thank Dr A. E. Oxford for helpful discussion and advice during the conduct of this work.

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The Amino Acid Composition of *Sarcina lutea* Grown on Different Media

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SUMMARY: The free amino acid composition of the 75 % ethanol extracts of a strain of *Sarcina lutea* varied considerably depending on the composition of the growth medium. Differences in hydroxyproline content were particularly significant. The amino acid composition of the ethanol-extracted cell residues remained constant.

In the course of some enzyme studies on acetone-dried *Sarcina lutea*, paper chromatography was used to identify amino acid reaction products, and it was observed that free amino acids diffused out of the organisms into the suspending medium. After spraying the chromatograms with ninhydrin, a bright orange spot due to hydroxyproline was particularly prominent. A qualitative examination of the free amino acids inside the organisms was undertaken, and comparisons were made between organisms grown on various media.

EXPERIMENTAL

Organism. *Sarcina lutea* was obtained from the Medical Research Council's Unit for Chemical Microbiology, Cambridge.

Culture media. A casein digest was prepared according to the method of Gladstone & Fildes (1940) for 'casein hydrolysate A'. The casein hydrolysate was stored in a dark bottle at +2°. Casein digest agar contained: yeast extract (Marmite Food Extract Co. Ltd., London, E.C. 3), 2 g.; Oxoid Lab-Lemco (Oxo Ltd., London, E.C. 4), 1 g.; NaCl, 5 g.; casein digest, 35 ml.; agar, 20 g.; distilled water to 1 l. Nutrient agar contained: proteose peptone (Difco Laboratories Inc., Detroit 1, Michigan), 5 g.; yeast extract (Marmite), 2 g.; Oxoid Lab-Lemco, 1 g.; NaCl, 5 g.; agar, 20 g.; distilled water to 1 l. 'Hydrolysed peptone agar' was made in the same way as nutrient agar except that the proteose peptone was replaced by an equivalent amount of acid hydrolysed peptone. 'Nutrient broth', used only for initial inocula, was made as for nutrient agar, but with omission of the agar. Glucose (10 g./l. when added) was autoclaved separately.

Cultivation and harvesting. The organism, *Sarcina lutea*, was maintained on nutrient agar slopes; it was inoculated, when required, into 2 ml. volumes of nutrient broth in bijoux bottles and incubated for 18-20 hr. (overnight) at 25°. The contents of one bijoux bottle were used to surface-inoculate 200 ml. solid medium contained in a Roux bottle. The organisms were harvested after 3 days of growth at 25°, physiological saline being used to wash the organisms

from the surface of the agar. The organisms were centrifuged for 20 min. at 5000 g, were resuspended (once) in saline and again centrifuged down. The packed organisms were then coagulated in acetone, filtered off, dried *in vacuo* and weighed.

Preparation of extracts of dried organisms. Aqueous ethanolic extracts were prepared and treated with chloroform as described by Lindan & Work (1951). Acetone-dried cells (200–250 mg.) were extracted with three successive 10 ml. batches of 75 % (v/v) ethanol in water with continuous shaking at room temperature (*c.* 18°) for 24 hr. The extracted residues of the organisms were hydrolysed by acid according to the procedure of Work & Dewey (1953), and the solutions were electro-dialysed using the apparatus described by Work (1950).

Nitrogen estimations were carried out by the micro-Kjeldahl method.

Paper chromatography. Chromatograms were made using Whatman no. 4 paper ($22\frac{1}{2} \times 18\frac{1}{4}$ in.) with phenol (ammonia atmosphere) as first solvent, and butanol + acetic acid (Campbell, Work & Mellanby, 1951) as second solvent. Ninhydrin-reacting spots were identified by reference to marker squares with known amino-acid mixtures; the strengths of spots were arbitrarily estimated by eye after drying and spraying, scoring with numbers ranging from 1 to 10.

RESULTS

The yields of *Sarcina lutea* grown on the three different solid media with and without glucose are shown in Table 1. In all cases the presence of glucose stimulated growth; growth was best on casein digest + glucose. Under these conditions *S. lutea* formed thick and raised colonies firmly attached to the surface of the agar. Growth on the nutrient agar and hydrolysed peptone agar media was much thinner and the organisms were easily washed from the agar surface.

Table 1. *Yields of Sarcina lutea grown on various solid media*

Culture medium	Yield of organism (mg. dry wt./ Roux bottle)	Increase in yield of organism on addition of 1 % (w/v) glucose (%)
Nutrient agar	220	—
Nutrient agar + glucose	420	91
Hydrolysed peptone agar	250	—
Hydrolysed peptone agar + glucose	420	68
Casein digest agar	1000	—
Casein digest agar + glucose	1660	66

The ethanolic extracts of organisms from each medium after chloroform treatment comprised 14–18 % of the corresponding total dry matter (Table 2).

Qualitative amino acid composition of the ethanol-extracted residues

Chromatograms of acid hydrolysates of the ethanol-extracted residues of organisms grown on the six different media were remarkably similar (Table 3). The hydrolysates were all subsequently electro-dialysed and the neutral fractions on chromatography were again very similar to one another. No unusual amino acids were present.

*Qualitative amino acid composition of the ethanolic extracts
of organisms*

Samples of the 'extract' (100 μ l. equivalent to 3–5 μ g. N) were first examined on chromatograms to indicate the general amino acid distribution. Each extract was then electro dialysed and the contents of the three fractions: anode fraction, neutral fraction (centre compartment) and cathode fraction, were concentrated and retained for separate examination. The neutral fractions were chromatographed in excess (equiv. 10–15 μ g. N) to reveal neutral amino acids present in amounts too low to be detected in the whole (untreated) extract (before electro dialysis). The approximate relative amounts of the known amino acids are given in Table 3.

Table 2. *Distribution of dry matter between soluble and insoluble fractions of Sarcina lutea grown on various media*

Culture medium	Wt. of organisms extracted (mg.)	Wt. of insoluble residue (mg.)	Amount of residue as % total dry wt. (%)	Wt. of ethanolic extract (mg.)	Amount of extract as % total dry wt. (%)	Amount of lipid as % total dry wt. (by difference) (%)
Nutrient agar	220	167	76	37	16	8
Nutrient agar + glucose	220	160	73	34	15	12
Hydrolysed peptone agar	250	188	75	36	14	11
Hydrolysed peptone agar + glucose	220	137	62	36	16	22
Casein digest agar	220	175	79	40	18	3
Casein digest agar + glucose	200	105	52	34	17	31

Glutamic acid and alanine were the most abundant amino acids under all growth conditions; aspartic acid was equally abundant except when nutrient agar was used. With nutrient agar or hydrolysed peptone agar as growth media, a very pronounced orange spot due to hydroxyproline was present; this was absent from extracts of organisms grown on the casein digest medium. Chromatographic examination of the growth media confirmed the absence of hydroxyproline from the casein hydrolysate, whereas a peptone hydrolysate contained hydroxyproline as the most abundant amino acid; unhydrolysed peptone did not appear to contain any free hydroxyproline. *Sarcina lutea* appeared to contain more free amino acids when grown on the casein digest medium; this was the most favourable medium for growth (see Table 1). Under these growth conditions appreciable amounts of proline were present in the ethanolic extracts, in contrast to trace amounts under the other growth conditions. Also of interest is the low amount of the basic amino acids in the extracts except when casein digest was the growth medium.

Compounds other than the common amino acids were found in the ethanolic extracts of *Sarcina lutea*. Organisms grown on nutrient agar and hydrolysed

Table 3. *Amino acids in Sarcina lutea grown on various media*

Culture medium	Fraction examined	Aspartic acid	Glutamic acid	Glycine + serine	Threonine	Alanine	Tyrosine	Valine	Phenylalanine	Leucines	Proline	Hydroxyproline	Basic amino acids	Asparagine	Glutamine
Nutrient agar	Whole extract	3	10	1	0	6	0	0	0	0	0	10	1	0	2
	Neutral fraction	—	—	8	2	10	0	1	0	1	0	10	—	2	4
Nutrient agar + glucose	Whole extract	3	10	2	0	6	0	2	0	0	0	10	1	0	2
	Neutral fraction	—	—	7	0	10	0	6	0	1	1	10	—	4	10
Hydrolysed peptone agar	Whole extract	10	10	2	0	2	0	0	0	0	0	8	1	0	2
	Neutral fraction	—	—	10	0	10	0	1	0	1	2	10	—	0	10
Hydrolysed peptone agar + glucose	Whole extract	9	10	1	0	8	0	1	0	0	0	5	1	0	1
	Neutral fraction	—	—	2	0	10	0	2	0	0	0	10	—	0	1
Casein digest agar	Whole fraction	8	10	2	0	9	0	2	0	6	3	0	3	0	0
	Neutral fraction	—	—	2	0	10	3	7	3	9	10	0	—	0	4
Casein digest agar + glucose	Whole extract	8	10	2	0	8	0	3	0	6	3	0	4	0	0
	Neutral fraction	—	—	5	0	10	1	7	1	9	10	0	—	0	0
Any of the above six media	Acid hydrolysate of extracted cell residue	7	10	6	5	10	5	10	6	10	7	0	6	—	—

Arbitrary figures ranging from 1 to 10 indicate the approximate relative strengths of the amino-acid spots on the chromatograms.

peptone agar media contained glutathione, glutamine and asparagine. Acid labile substances, probably peptides, were also present, the most conspicuous spot, which disappeared on hydrolysis, was found 'under alanine'. Another spot, which ran very fast in phenol but only slowly in butanol+acetic acid, was also probably a peptide. Other weak spots, which disappeared on hydrolysis, were also probably due to peptides. The presence of β -alanine in the extracts was suspected from its known chromatographic behaviour and characteristic bright blue colour with ninhydrin, but was not thoroughly confirmed. The bacterial amino acid $\alpha\alpha'$ diaminopimelic acid, not found in acid-hydrolysates of Gram-positive cocci (Work & Dewey, 1953), was not found in any of the extracts or extracted residues of *S. lutea*.

Examination of the contents of the anode and cathode fractions with and without hydrolysis failed to reveal any further useful information.

DISCUSSION

The above qualitative examination of the amino acids in *Sarcina lutea* shows that the composition of the free amino acid pool depends on the composition of the growth medium. A similar dependence was observed by Gale (1947) in the case of *Streptococcus faecalis*, and by Fernell & King (1953) for *Escherichia coli* and *Bacillus subtilis*. The latter workers disintegrated the bacteria before examining any soluble cell fractions. The free amino acids of a number of bacteria were also investigated by Taylor (1947); specific decarboxylase preparations were used to estimate the concentrations of glutamic acid, arginine, ornithine, lysine, histidine and tyrosine.

Attempts were made to estimate hydroxyproline in extracts of *Sarcina lutea* by the method of Troll (1953), but it was not found to yield reproducible results since other components of the extracts interfered. The high hydroxyproline content of extracts of *S. lutea* grown on media other than the casein hydrolysate contrasts sharply with its complete absence from the acid-hydrolysed residues under all growth conditions. It would seem that *S. lutea* can absorb hydroxyproline in large amounts from a suitable growth medium, and may hydrolyse peptone to liberate free hydroxyproline, but does not incorporate it into cellular protein.

In contrast to the variations in the ethanolic extracts of *Sarcina lutea*, the amino acid composition of the ethanol-extracted residues is constant irrespective of the growth conditions. Stokes & Gunness (1946) have stated that the amino acid composition of an organism is a stable and characteristic property of the cell under fixed conditions of growth. Freeland & Gale (1947) studied the amino acid composition of certain bacteria and yeast and found that the amino acid composition of the protein of *Escherichia coli* and *Aerobacter aerogenes* was unaffected by widely varying growth conditions. Work (1949) also observed that the amino acids of the insoluble cell residues of *Corynebacterium diphtheriae* grown on a casein digest or a synthetic amino acid medium were the same.

Sarcina lutea is capable of absorbing a wide variety of free amino acids in

varying proportions depending on the medium on which it is growing; it appears, however, to have a specifically selective mechanism for incorporating amino acids, from those already absorbed, into its cellular protein.

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Interaction of Viruses and Cells. Organized by F. C. BAWDEN and G. PENSO. 110 pp., 67 illus. Price 15s.
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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its Nineteenth General Meeting in the University of Reading on Tuesday and Wednesday, 14 and 15 September 1954. The following communications were made:

COMMUNICATIONS

A Group of Luminous and Non-Luminous Bacteria from the Intestine of Flatfish. By J. LISTON (*Torry Research Station, Aberdeen*)

A single well-defined group of organisms predominates in the intestine of skate and lemon sole from the North Sea. The group contains both luminous and non-luminous strains which share many properties in common. All are pleomorphic Gram-negative, psychrophilic, non-spore forming rods which ferment glucose and maltose (usually producing acid and gas), do not liquefy gelatin and reduce nitrates and trimethylamine oxide. They are more or less weakly motile. They will not grow at 30°, and are killed by exposure to 37° for 2 hr.

They have been subdivided into three groups. Group 1 is made up of organisms which produce light and characteristically carry out a late (7–21 days at 20°) fermentation of lactose. They are very similar to *Photobacterium phosphorescens*. Group 2 consists of frankly motile (more than 10% of the cells) non-luminous strains. Cells of these strains are less pleomorphic than those of either of the other groups and tend to assume the form of curved rods. These strains grow anaerobically on nutrient agar. Group 3 contains the bulk of the non-luminous strains which are closely similar in properties to the luminous group. They do not ferment lactose, however, and differ in their colony appearance on glycerol-sea-water agar from luminous strains. This group is subdivided into two sections on the basis of starch fermentation.

The antibiotics sensitivity patterns indicate that all these organisms are vibrios, and this is supported by their morphology and their preference for alkaline media. Because of this and the fact that the bulk of the strains in this group are non-luminous, it is obvious that neither *Photobacterium phosphorescens* nor *Bacterium phosphoreum* is a suitable binomial for these organisms.

The Conversion of α -Aminolaevulinic Acid to Porphyrins by Photosynthetic Bacteria. By JUNE LASCELLES (*Department of Biochemistry, University of Oxford*)

Some members of the photosynthetic, non-sulphur group of bacteria (Athiorhodaceae) excrete porphyrins into the medium during growth; this may be associated with the biosynthesis of bacteriochlorophyll. Cell suspensions of a member of this group, *Rhodospseudomonas spheroides*, have been used to study porphyrin formation from glycine and α -ketoglutaric acid and from δ -amino-

laevulinic acid. The latter compound and the monopyrrole, porphobilinogen, have been suggested by Shemin, Neuberger and Rimington as intermediates in the synthesis of protoporphyrin by animals.

The cells convert glycine and α -ketoglutarate (or succinate) to porphyrins only if incubated anaerobically in the light; Mg^{2+} , Mn^{2+} , NH_4^+ and an oxidizable substrate are required. Approximately 0.2 μ mole porphyrin (mainly coproporphyrin III) is produced by 1 mg. dry weight cells in 24 hr.; the overall yield is about 20% of the theoretical value.

δ -Aminolaevulinic acid (generously provided by Dr A. Neuberger and Mr J. J. Scott) replaces both glycine and α -ketoglutarate for porphyrin formation. When incubated in the light in phosphate buffer the cells convert it almost quantitatively to porphyrins and require the addition of Mg^{2+} only. Porphobilinogen (identified by reaction with Ehrlich's reagent) is formed during the conversion. In the simple system containing phosphate and Mg^{2+} , δ -aminolaevulinic acid gives rise mainly to coproporphyrin III (95%) and uroporphyrin I (4%) with traces of other porphyrins. Porphyrins are also formed from δ -aminolaevulinic acid in the presence of iron salts (Fe^{2+} or Fe^{3+}), whereas these ions almost completely suppress porphyrin formation from glycine and α -ketoglutarate. In mixtures containing δ -aminolaevulinic acid, Fe^{3+} , Mg^{2+} and fumarate, or other oxidizable substrates, protoporphyrin (20–30% of the total porphyrins) is formed as well as copro- and uroporphyrins. δ -Aminolaevulinic acid is also converted to porphyrins aerobically in the dark; Mg^{2+} and fumarate are required. Proto-, copro- and uroporphyrins are formed in the same ratio in these aerobic conditions without iron as anaerobically in the light with iron present.

The Flavins of some Micro-organisms. By J. L. PEEL (*A.R.C. Unit for Microbiology, University of Sheffield*)

The quantitative distribution of flavins in eighteen species of bacteria, of various metabolic types, has been examined using a method based on electrophoretic separation. The total flavin contents of eight aerobically grown species varied from 40 μ mole/g. dry wt. (*Micrococcus lysodeikticus*) to 290 μ mole/g. dry wt. (*Pseudomonas aeruginosa*). All but two of the anaerobically grown species contained between 120 (*Desulphovibrio desulphuricans*) and 570 (*Clostridium butyricum*) μ mole/g. dry wt. The other two organisms, *C. kluyveri* and LC1, a metabolically similar organism isolated from sheep rumen, contained much larger amounts (2860 and 1480 μ mole/g. dry wt. respectively). High flavin contents could not be correlated with aerobic growth, but were found to be associated with the production of higher volatile fatty acids.

Using yellow fluorescence under u.v. light as the definitive test, no flavin compounds have been found in these organisms other than riboflavin (or possibly riboflavinyl glucoside), flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). The proportion of free riboflavin was too small to permit confirmatory tests, and only exceeded 8% of the total flavin in one case (14% with an organism isolated from silage). FMN and FAD have been

separated from every organism examined, and in each case their identity has been checked by chromatography in four different solvents and by the use of D-amino-acid oxidase. The relative amounts of FMN and FAD varied from 27% FAD, 70% FMN (*Lactobacillus helveticus*) to 77% FAD, 23% FMN (*Desulphovibrio desulphuricans*). FAD was usually predominant, especially in the fatty acid forming species (e.g. *Clostridium kluyveri*, 71% FAD, 27% FMN).

Some Factors affecting Lactase Activity in *Saccharomyces fragilis* Jörgensen. By A. DAVIES (*Medical Research Council Unit for Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

The factors affecting lactase formation in logarithmically growing *Saccharomyces fragilis* have been studied using a continuous culture technique (Monod, 1950; Novick & Szilard, 1950), lactase activity being measured by fermentation of the products of lactose hydrolysis after treatment of the yeast with cetyltrimethylammonium bromide (CTAB).

Lactase formation is controlled mainly by the sugar concentration in the medium. With glucose concentrations of 0.001% or less, lactase contents up to 10 units/mg. dry weight (1 unit equals amount of enzyme hydrolysing 1 μ mole lactose in 1 hr.) are observed; at concentrations of 0.1% or higher enzyme formation is completely suppressed. This effect is similar to that found for invertase formation (Davies, 1954). In media containing 0.001% or less of lactose or galactose the lactase content is about 110 units/mg.; this is reduced to 39 and 28 units/mg. in 0.05% galactose and 0.01% lactose respectively. The mean generation time of the cells, the concentration of ammonia in the culture and variations in the amount of growth factors have no specific effects on the enzyme formation.

When grown in lactose or galactose the rate of hydrolysis of lactose by intact cells is only slightly greater than the rate of fermentation (which is the same as that of glucose) and appears to be independent of the lactase content as measured by the CTAB method. CTAB-treated cells have lactase activities 10 to 200 times greater than similar intact cells.

Intact cells grown in media containing glucose are unable to ferment or hydrolyse lactose; nevertheless, treatment with CTAB reveals a lactase content which may be as high as 10 units/mg.

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A Method for Studying the Metabolism of Fungi in the Warburg Apparatus using Spore Suspensions. By MOIRA E. K. HENDERSON (*The Macaulay Institute for Soil Research, Craigiebuckler, Aberdeen*)

This technique was developed with the aim of overcoming the difficulties usually encountered when experimenting with fungi in the Warburg apparatus,

e.g. the preparation of uniform suspensions and the high rate of endogenous respiration.

Hormodendrum cladosporioides and *Penicillium* sp., both of which have non-wettable spores, were used for most of the work. Strips of cellophane laid on potato-dextrose-agar in Petri dishes were streaked with spores of the fungi and after 7 days growth at 22° the strips were removed. Spore suspensions were prepared by shaking the strips in 1/1000 solution of Tween 80, which made the spores suspend uniformly. The majority of the spores were thus removed, while the mycelium adhered to the cellophane. The spores were centrifuged off, washed twice in distilled water and finally suspended in distilled water. One ml. of suspension was added to each Warburg flask along with mineral salts solution, 0.02–0.08 % (w/v) Difco yeast extract, buffer and KOH. The spores were incubated overnight in the Warburg and the substrates were tipped in in the morning when readings were commenced. All operations were carried out under sterile conditions.

Points recommending the method are: (a) the whole organism is being studied; (b) good uniformity exists between vessels containing equal aliquots from the same spore suspension; (c) the rate of endogenous respiration was always reasonably low and the addition of a utilizable substrate produced an immediate and marked increase in the rate of oxygen uptake. The technique is limited to fungi which produce large masses of easily detachable spores.

Evidence for the Unity of two of the Postulated Chromosomes in *Escherichia coli*, K12. By R. C. CLOWES AND D. ROWLEY (*The Wright-Fleming Institute of Microbiology, St Mary's Hospital Medical School, London, W. 2*)

The variations in the sugar fermentation characters among the recombinants arising from matings of various K 12 strains have been shown to be influenced by the selected markers carried by both F+ and F– parents. Among the variations observed were differences in the fermentation of Xylose and Mannitol between the methionine+ and methionine– recombinants arising from a mating, using as the F+ parent a doubly auxotrophic strain, one requirement being methionine.

This implies a positive linkage between the M locus and the Xyl. and Mtl. markers. The hypothesis of Watson & Hayes (1953, *Proc. Nat. Acad. Sci., Wash.* **39**, 116) elaborated by Hayes (1953, *Cold Spr. Harb. Symp. Quant. Biol.* **18**, 75) requires that these markers be allocated to two different chromosomes. That they are shown to be linked can be explained only by their location on the same chromosome and suggests that the remaining two linkage groups may be shown to have a similar relationship.

A Mouse Virulence Factor among *Escherichia coli* Strains. By D. ROWLEY (*Wright-Fleming Institute of Microbiology, St Mary's Hospital Medical School, London, W. 2*)

The virulence for mice of twenty different strains of *Escherichia coli* has been tested by intraperitoneal injection of dilutions of a 14 hr. culture, together with 1.7 % hog gastric mucin. Most of the strains are avirulent and have an

LD50 of at least 10^7 organisms, a few strains however have an LD50 of around 10^3 – 10^4 organisms. *In vivo* growth curves of several strains have been constructed from peritoneal washings and show that the virulent strains multiply rapidly from small inocula, whereas the avirulent strains can only multiply for a few hours and are then rapidly eliminated. The virulent strains are much more resistant than the other strains to the *in vitro* bactericidal action of complement-containing sera. It is suggested that resistance or otherwise to this *in vitro* effect is a major determinant of virulence under these conditions.

Transfer of Motility by *Escherichia coli* K12 to *E. coli* B. By G. FURNESS AND D. ROWLEY (*The Wright-Fleming Institute of Microbiology, St Mary's Hospital Medical School, London W. 2*)

A motile strain of *E. coli* K 12 was mixed with a streptomycin resistant mutant of the non-motile strain of *E. coli* B and incubated at 37° for 4 hr.

When loopfuls of this suspension were streaked on soft gelatin agar containing streptomycin, motile flares could frequently be seen.

Controls were always negative.

Of thirty-five motile isolates all had the marker characters of the *E. coli* B parent so it was impossible to show linkage with any other character.

The Site of Action of Polymyxin on *Bacillus megaterium*. By B. A. NEWTON (*Medical Research Council Unit for Chemical Microbiology, University of Cambridge*)

Previous studies (Few & Schulman, 1953; Newton, 1953) have shown that the addition of polymyxin to washed cell suspensions of sensitive organisms causes a release of soluble constituents from the cells; it was suggested by these authors that polymyxin combines with and disorganizes structures within the bacterial cell wall which are responsible for the maintenance of the osmotic equilibrium of the cell. A more direct approach to the problem of the site of action of polymyxin has been made possible by studies on protoplasts using a fluorescent derivative of the antibiotic (DANSP) prepared by coupling 1-dimethylaminonaphthalene-5-sulphonyl chloride with the γ -amino group of $\alpha\gamma$ -diaminobutyric acid radicals in the polymyxin molecule. DANSP is rapidly absorbed by washed cells of *Bacillus megaterium* and growth tests have shown that this derivative has approximately the same bactericidal activity as the untreated antibiotic. Mechanical disintegration of cells pretreated with a bactericidal concentration of DANSP has shown that 90% of the fluorescent compound is associated with a small particle fraction and 10% with the cell walls.

Controlled lysozyme treatment of *Bacillus megaterium* suspended in 0.2 M sucrose results in a depolymerization of the cell wall leaving an intact sub-cellular unit or 'protoplast' (Weibull, 1953). Protoplasts prepared from DANSP-treated *B. megaterium* fluoresced strongly in ultraviolet light and fluorescence photomicrographs showed that the derivative is associated with the protoplast membrane. When fluorescent protoplasts were subjected to

supersonic vibration the protoplast structure was destroyed; the fluorescent material in the supersonic disintegrate was found to be associated with a small particle fraction formed from the protoplast membrane.

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Stimulation of Pigmentation and Sporulation in Fungi by Metabolites of Actinomycetes. By ERNA GROSSBARD (*Yale University, U.S.A., and the University of Leeds*)

In antifungal paper disk assays of metabolites of various Actinomycetes certain stimulation effects occurred within a concentric circle adjoining the zone of inhibition. Pigmentation was intensified in certain strains of *Verticillium dahliae*, in *Helminthosporium victoriae* and *Fusarium oxysporum* f. *lycopersici*. The latter normally fails to form a pigment on Czapek-Dox medium, but in response to antibiotic metabolites of four species of *Streptomyces* a pigment (probably lycopersin) appeared, while a considerable intensification occurred on media optimal for lycopersin production. *Colletotrichum atramentarium* responded to the same antibiotics mainly by an acceleration in the maturing and by a greater density of stromata. Other metabolites promoted sporulation in *Ceratostomella ulmi* and *Streptomyces scabies*. When antibiotic activity was removed by adsorption, the characteristic stimulation effects disappeared. On the other hand, inhibitors such as mercuric chloride and 8-hydroxy-quinoline (only in acid solutions) induced pigment formation in *Fusarium* and mercuric chloride considerably increased coremia formation in *Ceratostomella*. When the metabolites or other inhibitors were used below threshold concentrations, stimulation effects were absent. Thus, they may be regarded as a response to the adverse conditions leading up to growth inhibition. This hypothesis is supported by the observation that in shake cultures of *Fusarium* in Czapek-Dox, pigmentation occurred in dried-up, starved mycelial fragments adhering to the walls of the flasks. Possibly, in agar assays degradation products accumulate at the periphery of the zone of inhibition providing some specific materials required for pigmentation and/or formation of spore-bearing structures. However, some inhibitors, such as actidione, do not affect pigmentation in *Fusarium* and stromata formation in *Colletotrichum*. Furthermore, the pattern obtained with the Actinomycetes, though similar, is not identical with that of other inhibitors. Therefore, it is possible that Actinomycete metabolites contain in addition to inhibitory also specific stimulatory substances.

Followed by a Discussion Meeting on

PRINCIPLES OF MICROBIAL CLASSIFICATION

Introduction: the Philosophy of Classification. By S. T. COWAN (*National Collection of Type Cultures, Colindale*)

Nomenclature, the Handmaid of Classification. By G. C. AINSWORTH (*Department of Botany, University College, Exeter*)

General Morphology. By T. GIBSON (*Edinburgh and East of Scotland College of Agriculture*)

The Value of Cytological Studies in Elucidating Natural Relationships among Bacteria. By K. A. BISSET (*Bacteriology Department, University of Birmingham*)

The Impact of Genetics. By G. PONTECORVO (*Department of Genetics, University of Glasgow*)

Considerations of General Physiology. By S. R. ELSDEN (*Department of Microbiology, University of Sheffield*)

Methods for Determining Biochemical Activities of Micro-organisms as applied to Classification. By PATRICIA H. CLARKE (*Department of Biochemistry, University College, London*)

Bacterial Toxins and Classification. By C. L. OAKLEY (*Department of Bacteriology, University of Leeds*)

Nutritional Characters. By B. C. J. G. KNIGHT (*Department of Microbiology, University of Reading*)

Host-Parasite Relationships. By G. C. AINSWORTH (*Department of Botany, University College, Exeter*)

The Classification of Viruses. By F. O. HOLMES (*Rockefeller Institute, New York*). **The Classification of Viruses.** By C. H. ANDREWES (*National Institute for Medical Research, Mill Hill, London*). **The Classification of Viruses.** By F. C. BAWDEN (*Rothamsted Experimental Station, Harpenden*)

The Use of Serology in the Classification of Micro-organisms. By P. M. FRANCES SHATTOCK (*Department of Microbiology, University of Reading*)

Bacteriophage and Bacterial Classification. B. A. D. STOCKER (*Lister Institute, London*)

Summing-up. By N. W. PIRIE (*Rothamsted Experimental Station, Harpenden*)

Summaries of the main contributions and discussions will appear elsewhere in this *Journal*.

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